ROLE OF DELTA SUBUNIT-CONTAINING $GABA_A$ RECEPTORS IN HIPPOCAMPUS TONIC INHIBITION AND EPILEPTOGENESIS WITHIN TRANSGENIC MOUSE MODELS

A Dissertation

by

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ABSTRACT

Epilepsy is associated with marked alterations in the structure and function of $GABA_A$ receptors in the hippocampus, a key structure for the genesis of epilepsy. Two types of inhibition are mediated via distinct $GABA_A$ receptors. Phasic inhibition results from the synaptic γ_2-containing receptors, whereas tonic inhibition is primarily mediated by the continuous activation of δ-containing, extrasynaptic receptors by ambient GABA present in the extracellular fluid. The δ-subunit receptors exhibit greater sensitivity to neurosteroid potentiation through positive allosteric modulation. The abundance of δ-subunit and the extent of tonic inhibition are altered by physiological and pathological neuroendocrine conditions. However, the precise functional impact of δ-subunit on inhibition in the hippocampus and epileptogenesis remain poorly understood.

The main objective of this dissertation research was to understand the role of δ-subunit-containing $GABA_A$ receptors in the hippocampus dentate gyrus in mediation of tonic inhibition and epileptogenesis using a combination of electrophysiological, behavioral, and pharamcological techniques. We sought to understand the contribution of δ subunit to GABAergic inhibition and network excitability. We incorporated a perimenstrual model of catamenial epilepsy in which female mice experience acute neurosteroid withdrawal. The correlates of receptor plasticity and function were then examined. Furthermore, the structure-activity relationship of neurosteroids at extrasynaptic $GABA_A$ receptors was investigated in conducting the tonic inhibition. Alterations to hippocampus epileptogenesis of the δ-subunit knockout mouse as a model for hyperexcitability and susceptibility to seizures was also explored. Overall, these studies reveal a unique and novel role for δ-subunit-containing $GABA_A$ receptors as key modulators of tonic inhibition and excitability in the brain. These extrasynaptic receptors may represent new therapeutic targets for the control of epileptic conditions.

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NOMENCLATURE

3α-HSOR	3α-Hydroxysteroid oxidoreductase
3β-AP	3β-hydroxy-5α-pregnan-20-one
5β-AP	3α-hydroxy-5β-pregnan-20-one
δKO	$GABA_A$ receptor δ-subunit knockout
AD	Androstanediol (5α-androstan-3α,17β-diol)
ALFX	Alfaxolone (3α-hydroxy-5α-pregnan-11,20-dione)
AN	Androsterone
AP	Allopregnanolone (3α-hydroxy-5α-pregnan-20-one)
APV	D,L-2-amino-5-phosphonovaleric acid
CA1	Cornu Ammonis area 1
CA3	Cornu Ammonis area 3
CNS	Central nervous system
DGGC	Dentate gyrus granule cell
DNQX	6,7-dinitroquinoxaline-2,3-dione
DS2	4-chloro-*N*-[2-(2-thienyl)imidazo[1,2-a]pyridin-3-yl]benzamide
ETIO	Etiocholanolone
GABA	γ-aminobutyric acid
$GABA_AR$	γ-aminobutyric acid type A receptor
GBZ, GZ	Gabazine, SR-95531 (4-[6-imino-3-(4-methoxyphenyl) pyridazin-1-yl] butanoic acid hydrobromide)
GNX	Ganaxolone (3α-hydroxy-3β-methyl-5α-pregnan-20-one)
IPSC	Inhibitory postsynaptic current
ORG-20599	2β,3α,5α-21-Chloro-3-hydroxy-2-(4-morpholinyl)pregnan-20-one
NMDA	*N*-Methyl-D-aspartate
THDOC	Allotetrahydrodeoxycorticosterone (3α,21-dihydroxy-5α-pregnan-20-one)
THIP	Gaboxadol (4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridine-3-ol
TLE	Temporal lobe epilepsy
TTX	Tetrodotoxin
QX-314	*N*-(2,6-Dimethylphenylcarbamoylmethyl)triethylammonium bromide

CHAPTER I
BACKGROUND AND INTRODUCTION*

I.1 $GABA_A$ Receptor Subunit Composition and Structure

$GABA_A$ (γ-aminobutyric acid type A) receptors are composed of a pentamer of subunits, and each of subunit has four transmembrane domains (M1-M4) (**Figure 1**). The pentamer within the neuronal plasma membrane forms a selective channel for chloride ions when gated and opened by GABA. The second transmembrane domain lines the internal wall of the channel pore. There are at least 19 variants of $GABA_A$ receptor subunits (α1-6,β1-3,γ1-3,δ,ε,θ,π,ρ1-3). $GABA_A$ receptors are typically composed of two α subunits, 2 β subunits, and one γ subunit, which accounts for approximately 90% of $GABA_A$ receptors (Sieghart, 2006). The δ subunit can be assembled as the fifth subunit instead of γ, and there is high incidence of its occurrence within the cerebellum, thalamus, and hippocampus (Pirker et al., 2000). The functional stoichiometry forming the synaptic channel pore is 2α:2β:1γ (Baumann et al., 2002; Ernst et al., 2003). Receptors of extrasynaptic α4βδ isoform assemble functional channels with a stoichiometry of 2α:2β:1δ (Shu et al., 2012). Due to the large degree of heterogeneity in pentamer isoforms, the classification of underlying $GABA_A$ receptor physiology and pharmacology has become an expansive field of study.

Synaptic $GABA_A$ receptors are primarily γ_2-containing channels. The α-subunit expression within synaptic receptors varies, but they are typically benzodiazepine sensitive (Hajos et al., 2000). Synaptic receptors respond to vesicular release of GABA across the synapse, resulting in transient, phasic events. Extrasynaptic receptors are most often characterized by the inclusion of δ subunit rather than γ_2 together with α and β2/3. Extrasynaptic receptors respond to low levels of ambient GABA in continuous opening of channels known as tonic inhibition. This inhibition results in prolonged hyperpolarization and shunting of excitatory activity. Tonic inhibition plays a unique role in controlling hippocampal seizures by setting the baseline excitability (Brickley and Mody, 2012). The α-subunit isoforms contribute to phasic or tonic inhibition differentially in hippocampal neurons (Prenosil et al., 2006). The $\alpha_4\beta\delta$-containing receptors are found within

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dentate gyrus granule cells (DGGC) and thalamic relay neurons, whereas the expression of $\alpha_6\beta\delta$ is pervasive within and exclusive to cerebellar granule cells.

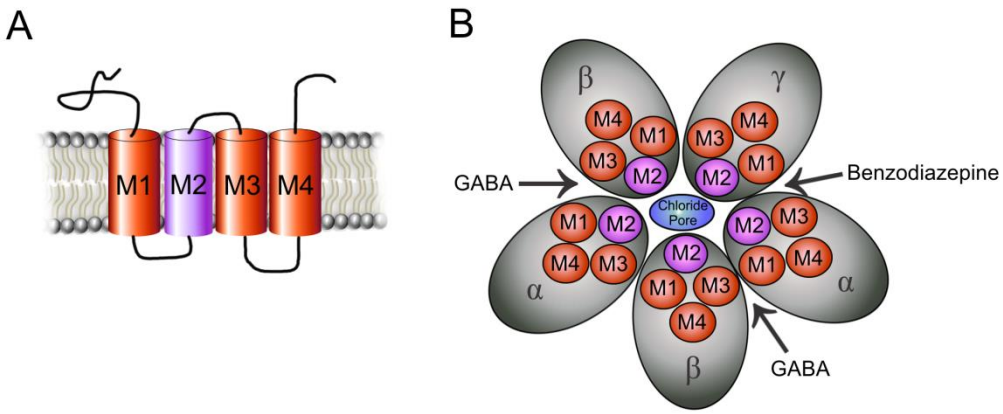


Figure 1. $GABA_A$ receptor structure and subunit arrangement. $GABA_A$ receptors are heteropentamers made up from 19 known subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π, and ρ1-3) with an integral channel that is permeable to Cl^- ion. ***A***, A cross section of the intramembrane structure shows the Cl^- channel pore formed by M2 helical elements. Each subunit has four transmembrane segments, with both the amino and carboxy terminals located extracellularly. ***B***, A top view of $GABA_A$ receptor channel, illustrated as five cylinders arranged to form the Cl^- pore by the M2 domains. The receptor is pentameric, being composed of two α, two β, and one γ subunit stoichiometry. The δ subunit may be assembled in the place of γ. Binding of GABA in the two sites at the interface between α and β subunits opens the receptor-associated Cl^- channel.

Interneurons of the hippocampus may contain α1βδ receptors rather than containing α4 like the principal neurons within the dentate gyrus. In addition, α5βγ2 extrasynaptic receptors have been typified in CA1-3 neurons of the hippocampus (Persohn et al., 1992; Benke et al., 1997; Pirker et al., 2000; Wei et al., 2003; Sun et al., 2004; Mangan et al., 2005; Zheleznova et al., 2009). These profiles are common expression patterns, however much crossover exists in the variety of subunit assembly within brain regions and neuronal cell-type specificity.

In the hippocampal DGGCs, α4βδ receptors are expressed primarily extrasynaptically, whereas α1βγ2 and α2βγ2 receptors are located at synpatic sites. However, α1, α4, γ2, and δ subunits are all present in the extrasynaptic or perisynaptic location of neurons (Sun et al., 2004). According to immunostaining studies, a population of interneurons in the molecular layer of the dentate gyrus also express the δ subunit, but the α4 subunit is not present (Sun et al., 2004). These receptors have been found to coexpress and colocalize α1 and δ subunits extrasynaptically with different kinetics and pharmacology than α4/α6βδ receptors (Glykys et al., 2007). In addition, parvalbumin-positive interneurons in the subgranular zone of the granule layer have been shown to co-express α1 and δ (Milenkovic et al., 2013). Therefore, principal cells and interneurons in the hippocampus may have quite distinct extrasynaptic receptor composition and properties. Further investigations into β subunit-containing receptors have identified that β2 is a component of benzodiazepine-insensitive receptors contributing to extrasynaptic $GABA_A$ receptor currents in DGGCs, whereas β3-containing receptors are predominantly assembled in synaptic receptors associated with phasic current (Herd et al., 2008).

I.2 Neurosteroid Background

Neurosteroids are steroids synthesized within the brain with rapid effects on neuronal excitability. The term *neurosteroid* was coined in 1981 by the French endocrinologist Etienne-Emile Baulieu to refer to steroids that are synthesized de novo in the nervous system, independent of the peripheral steroidogenic endocrine glands (Baulieu, 1981). Subsequently, the term *neuroactive steroid* has been widely used to describe natural or synthetic steroids that rapidly alter the excitability of neurons by binding to membrane-bound receptors (Paul and Purdy, 1992; Kulkarni and Reddy, 1995; Reddy 2003a). From the pioneering work of Hans Selye, it has been known that naturally occurring steroids such as the ovarian steroid progesterone and the adrenal steroid deoxycorticosterone can exert anesthetic and anticonvuslant

actions (Selye, 1941). Recognizing that some steroids could produce acute central nervous system effects, researchers at the pharmaceutical company Glaxo identified the synthetic steroid alfaxolone as having anesthetic properties. Alfaxolone was found to enhance synaptic inhibition via binding to $GABA_A$ receptors in the brain (Scholfield, 1980; Harrison and Simmonds, 1984).

A major advance occurred when 5α-reduced metabolites of progesterone and deoxycorticosterone were found to enchance $GABA_A$ receptor function (Majewska et al., 1986; Kulkarni and Reddy, 1995). The anesthetic and anticonvulsant properties of progesterone and deoxycorticosterone, known since the time of Selye, are due to their conversion to allopregnanolone (3α-hydroxy-5α-pregnane-20-one, AP) and allotetrahydrodeoxycorticosterone (3α,21-dihydroxy-5α-pregnane-20-one, THDOC), respectively (Reddy, 2003b) (**Figure 2**). The androgenic neurosteroid androstanediol (5α-androstan-3α,17β-diol, AD) is synthesized from testosterone. There is compelling evidence that all the enzymes required for the conversion of the steroid hormone precursors to their active 5α-reduced metabolites are present in the brain, and therefore their synthesis from cholesterol can occur locally (Kulkarni and Reddy, 1995; Do Rego et al., 2009). Therefore, AP, THDOC, and androstanediol can be considered endogenous neurosteroids (Reddy, 2011). Since neurosteroids are highly lipophilic and can readily cross the blood-brain barrier, neurosteroids synthesized in peripheral tissues accumulate in the brain. Pregnenolone, its sulfate ester pregnenolone sulfate (PS), dehydroepiandrosterone (DHEA), and its sulfate ester, DHEAS, are also present in the brain and could elicit rapid effects in enhancing neuronal excitability as negative modulators of $GABA_A$ receptors.

$GABA_A$ receptors are responsible for the majority of inhibitory activity within the brain. Increasing evidence points to the vast array of potent effects that endogenous neurosteroids have on $GABA_A$ receptors in modulating brain inhibition (Reddy 2013a, b). $GABA_A$ receptors are membrane-bound, ligandgated ion channels which, when activated by GABA, hyperpolarize neurons through influx of negatively charged chloride ions in the adult brain. At birth, the chloride driving force is positive such that GABA transmission causes depolarization. As the brain matures, a negative shift in the chloride ion reversal potential alters the electrochemical gradient to allow for inward current flow upon $GABA_A$ receptor channel opening (Rivera et al., 1999). The hyperpolarizing current serves to reduce neuronal excitability and short-circuit action potential firing. Inhibitory $GABA_A$ receptors are heterogenic, possessing a high degree of

variation in their subunit composition and pharmacodynamics. $GABA_A$ receptor agonist and antagonist ligands each have distinct affinity and efficacy for receptor isoforms (Mitchell et al, 2008; Uusi-Oukari and Korpi, 2010). Inhibitory network control by $GABA_A$ receptors is essential to maintain proper neuronal function for motor actions, cognition, autonomic activity, and nearly every other function of the brain. For the purposes of this dissertation, discussion will be centered on neurosteroid and $GABA_A$ receptor interaction as it pertains to limbic structures, specifically the hippocampus.

The human brain contains approximately 100 billion neurons. An estimated 20-30% of the neurons within the central nervous system are GABAergic. Activation of neuronal $GABA_A$ receptors typically results in hyperpolarization, and thus, GABA is the major inhibitory neurotransmitter in the brain. In the general scheme of feed-forward and feed-back interactions between principal neurons and interneurons, inhibitory circuits play a critical role in regulating network excitability. By controlling spike timing and sculpting neuronal rhythms, inhibitory GABAergic interneurons play a key role in regulating neuronal circuits.

I.3 Neurosteroid Biosynthesis and Regulation in the Brain

Neurosteroids are synthesized *de novo* in the brain or derived in peripheral endocrine tissues from metabolism of classical steroids such as progesterone, deoxycorticosterone, and testosterone. Reduction reactions are catalyzed by 5α-reductase and subsequently 3α-hydroxysteroid oxidoreductase (3α-HSOR) (**Figure 2**). AP and THDOC are classified as pregnane steroids, and they are the most commonly studied endogenous neurosteroids (Belelli and Lambert, 2005). AP is synthesized from progesterone, and THDOC is synthesized from deoxycorticosterone. Pregnenolone, progesterone, and deoxycorticosterone are pregnane steroid precursors and also exhibit neuroactive effects. The androstane class of neurosteroids, including androstanediol (AD), androsterone (AN), and etiocholanolone (ETIO) are derived from testosterone (Reddy, 2004b; Kaminski et al., 2006; Reddy, 2008). Estradiol has neuroactive effects in modulating GABAergic substrates and regulation of neuronal plasticity to promote excitability, especially within the hippocampus (Herzog, 2009). Synthesis of estradiol can occur through the aromatase-driven modification of testosterone, which could serve as an endogenous regulator of neuronal excitability in men, as well as women (Herzog, 1999).

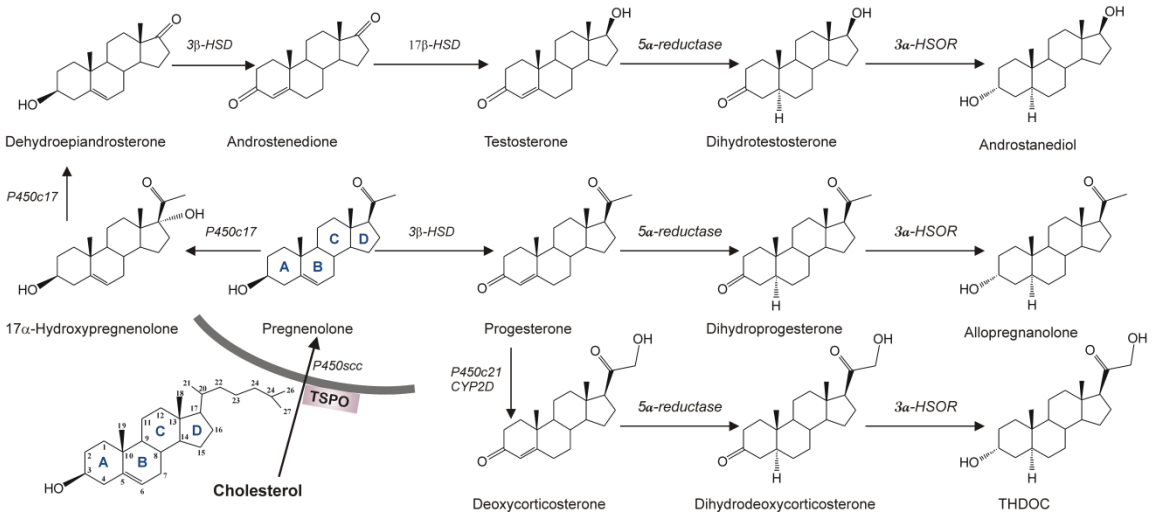


Figure 2. Biosynthetic pathways of prototypical endogenous neurosteroids. Enzymatic pathways for the production of three protoype neurosteroids allopregnanolone, THDOC, and androstanediol are illustrated from cholesterol and intermediate steroid precursors. Cholesterol is converted to pregnenolone by P450cc in the inner mitochondrial membrane. Pregnenolone is the precursor for progesterone and other neurosteroids. Progesterone, deoxycorticosterone, and testosterone undergo two sequential A-ring reduction steps catalyzed by 5α-reductase and (3α-HSOR) to form the 5α,3α-reduced neuroactive steroids. The conversion of progesteron, deoxycorticosterone, or testosterone into neurosteroids occurs in several regions within the brain. The 5α-reductase, 3α-HSOR, and other enzymes are present within the brain. *P450scc*: cholesterol side-chain cleavage enzyme, *3β-HSD*: 3β-hydroxysteroid dehydrogenase, *3α-HSOR*: 3α-hydroxysteroid oxidoreductase, *17β-HSD*: 17β-hydroxysteroid dehydrogenase, *P450c21*: cytochrome P450 21-hydroxylase

Neurosteroids reach neuronal $GABA_A$ receptors from the periphery or local brain tissue. Precursor steroids may enter the brain from the blood circulation and can be converted to neurosteroids (Agis-Balboa et al., 2006). Subcutaneous injection of progesterone rapidly elevates plasma and brain levels of AP in rodents, even in the case of animals in which the progesterone receptor gene is removed (Reddy and Mohan, 2011). The drug finasteride is used as a tool to inhibit endogenous neurosteroid biosynthesis, as it has strong specificity in blocking the activity of type II 5α-reductase conversion to their 5α-dihydro-reduced form (Reddy, 2010; Reddy and Ramanathan, 2012).

In addition to peripheral production and humoral delivery, it is clear that neurosteroids can be formed locally in the brain from steroid hormone precursors. 5α-Reductase activity has been identified in both neurons and glia in rodent and sheep brain within regions such as the neocortex and hippocampus (Melcangi et al., 1998; Petratos et al., 2000). 3α-HSOR is also widely expressed in the brain (Khanna et al., 1995). In humans, both enzymes have been found in neocortex and hippocampus (Stoffel-Wagner et al., 2000, 2003; Stoffel-Wagner, 2001). Taken together, all of the necessary enzymes required for steroid synthesis are region specific, cell specific, and available within the neural tissue, both in neurons and glia (Compagnone and Mellon, 2000; Porcu et al., 2009).

I.4 Neurosteroid Modulation of $GABA_A$ Receptors

The endogenous neurosteroids AP, THDOC, and androstanediol are positive allosteric modulators of $GABA_A$ receptors, which modify the natural affiinity and/or efficacy of GABA and thereby exert control over neuronal excitability. At concentrations in the range 10-500 nM, AP and THDOC enhance the activation of $GABA_A$ receptors by GABA (Harrison et al., 1987; Kokate et al., 1994; Reddy and Rogawski, 2002). At higher concentrations, these neurosteroids directly activate the receptor without the requirement of GABA agonist activity. The exact binding sites are not well understood; the location and maximal number of neurosteroid binding sites are not clearly established. Therefore, there is no validated assay for measuring the direct binding of neurosteroids to $GABA_A$ receptors. Alternative or allosteric binding assays are commonly used to study neurosteroid-$GABA_A$ receptor interactions. Unlike benzodiazepines (which enhance the channel open frequency) or barbiturates (which increase the channel open duration), neurosteroid positive allosteric modulation of $GABA_A$ receptors occurs in a hybrid

fashion, increasing both the frequency and duration of channel opening (Twyman and Macdonald, 1992; Hosie et al., 2007, 2009; Lambert et al., 2009; Ramakrishnan and Hess, 2010). Moreover, neurosteroids operate on a wider array of receptor isoforms than classical benzodiazepines. Thus, neurosteroids greatly enhance the probability of $GABA_A$ receptor chloride channel opening, thereby enhancing $GABA_A$ receptor-mediated inhibition.

Consistent with potentiation effects at the cellular level, endogenous neurosteroids like AP and THDOC are capable of sedative, anxiolytic, and anticonvulsant behavioral effects on the central nervous system (Reddy and Kulkarni, 1997; Reddy, 2003a, 2011; Reddy and Zeng, 2007). It is important to understand the systemic pharmacological effects of neuromodulatory agents. Structure-activity studies of neurosteroid potentiation of GABA-gated currents revealed unique structural features of neurosteroids (Harrison et al., 1987; Gee et al., 1988). A range of steroid structures have activity as positive modulators of $GABA_A$ receptors in agreement with the hydrophobic surface binding site model. Nevertheless, there are certain strict structural requirements for neruosteroid positive modulation. A hydrogen bond-donating 3α-hydroxy group on the steroid A-ring and a hydrogen bond-accepting group (typically a keto moiety) on the D-ring at either C20 of the pregnane steroid side chain or C17 of the androstane ring system are critical for positive modulatory activty at $GABA_A$ receptors (Purdy et al., 1990; Lambert et al., 2003). Structural configurations of α and β neurosteroid epimers have highly differential effects on $GABA_A$ receptor function, owing to the stereoselectivity of binding. The orientation of the C5 hydrogen group only modestly influences potency, such that 5β-epimers are less potent than the corresponding 5α-epimers (Kokate et al., 1994). Behavioral, electrophysiological, and pharmacological data substantiate that 5α-reduced steroids, but not 5β-reduced steroids possess a high level of enantiospecificity and selectivity in function of $GABA_A$ receptor modulation and anesthetic efficacy (Covey et al., 2000). However, these data have yet to be substantiated in functional studies of neurosteroid activity on extrasynaptic receptor currents.

Neurosteroids can act as autocrine factors in principal neurons or interneurons. Although neurosteroids are viewed as high potency modulators of $GABA_A$ receptors since they are effective at concentrations in the nanomolar range in aqueous solution, recent studies indicate that neurosteroid binding to the $GABA_A$ receptor is actually of low affinity (true membrane EC_{50}, ~1 mM) (Chisari et al., 2010). The high effective potency of neurosteroids results from

partitioning of the lipophilic steroids within the plasma membrane, such that the concentrations accessible to the receptor are orders of magnitude greater. Neurosteroids access the $GABA_A$ receptor from the lipohilic plasma membrane. The nonspecific accumulation and removal of the neurosteroids from the membrane are the major factors determining the rates of neurosteroid action when applied to cells via aqueous solution; rates of binding and unbinding are only secondary factors (Chisari et al., 2009). It is noteworthy that intracellular delivery through the plasma membrane is compatible with the autocrine mechanism discussed above, in which the neurosteroids act on $GABA_A$ receptors in the same neurons in which they are produced (Saalmann et al., 2007).

Endogenous neurosteroids often have low availability due to the rapid inactivation or conversion into other steroidal products. To overcome these limitations, synthetic neurosteroid analogs have been developed for therapeutic use (Reddy and Kulkarni, 2000; **Figure 3**). Analogs also serve as pharmacological tools to better understand specific ligand binding characteristics of neurosteroids based on their conserved lipophilic properties. Ganaxolone (3α-hydroxy-3β-methyl-5α-pregnan-20-one, GNX) is an analog of AP, which is altered to include a β-methyl group at the C3 position (Carter et al., 1997; Reddy and Woodward, 2004). GNX posesses sedative and anticonvulsant effects and positively modulates $GABA_A$ receptors allosterically, similar to AP (Reddy and Rogawski, 2010). It is the only neurosteroid analog thus far to be considered in human clinical trials for the treatment of epilepsy (Nohria et al., 2010). Alfaxalone is a general anesthetic that has been well classified in $GABA_A$ receptor binding characteristics. The synthetic derivative minaxolone has been shown to induce an extrasynaptic, GABAergic conductance, similar to AP at 1 μM concentrations (Mitchell et al., 2007). These analogs have the ability to potentiate $GABA_A$ receptors and induce sedative, anticonvulsant, anesthetic, or anxiolytic actions, very similar to the endogenous neurosteroids. Their positive interaction with receptors verifies that neurosteroid structural selectivity is reliant on conserved sites to bind and enhance GABAergic function.

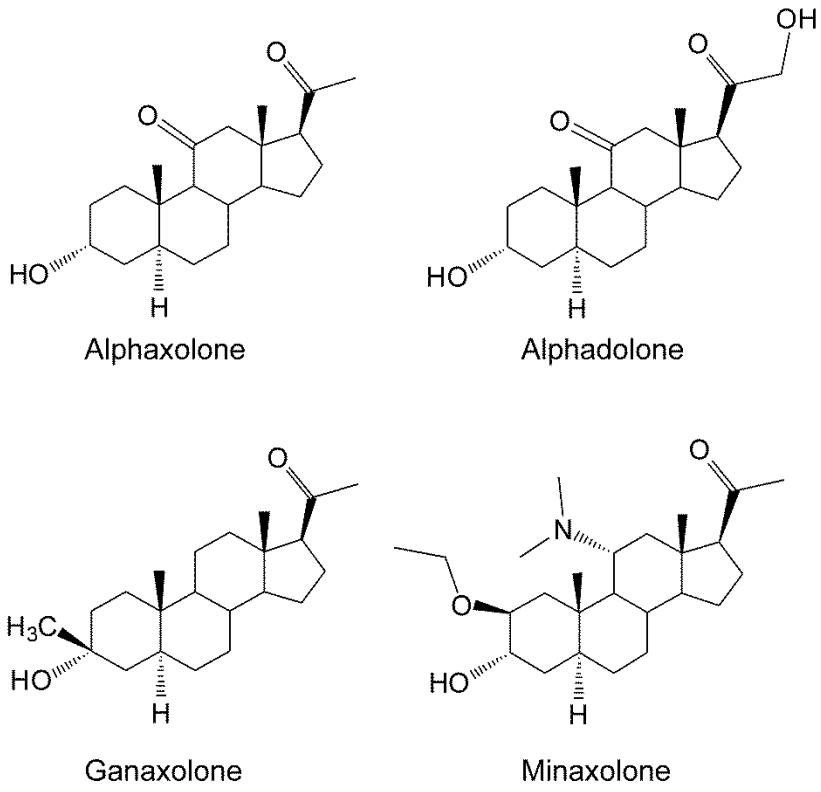


Figure 3. Chemical structures of synthetic neurosteroids. Alphaxolone (alfaxolone, ALFX) is an analog of AP in the addition of the C11 position ketone with a conserved C17 group. Alphadolone is an analog of THDOC in the addition of the C11 position ketone with a conserved C17 group. Ganaxolone is an analog of AP in the addition of the C3β-position methyl group, but retaining the 3α-hydroxyl structural feature. Minaxolone contains a C2 position ethoxyl group and the C11 position dimethylamino group. Ganaxolone is the only synthetic neurosteroid to that is currently undergoing testing in clinical trials for the modulation of seizures.

While neurosteroids are able to modulate both synaptic and extrasynaptic receptors (**Figure 4**), there are clear differencies in efficacy between γ-containing and δ-containing receptors (Pillai et al., 2004) as well as differences between the composition of α subunits (Belelli et al., 2002; see **Table 1**). While much has been determined concerning the subunit isoform influences on $GABA_A$ receptor properties, most comparative information has been uncovered using heterologous expression systems rather than native neurons. The δ-containing receptors produce a much higher maximal response to neurosteroids (Brown et al., 2002), and they possess unique functional actions at extrasynaptic and dendritic sites, where they respond to low levels of ambient GABA (Wei et al., 2003; Semyanov et al., 2004). δ-containing receptors participate in a distinctive role to control the excitability of the hippocampus and other neuronal regions by setting a baseline level of inhibitory current. Therefore, receptors that are attuned to extrasynaptic concentrations of GABA (low-efficacy) can be modulated by endogenous agents like neurosteroids that can finely adjust and potentiate tonic inhibition (Stell et al., 2003; Farrant and Nusser, 2005). Knockout studies reveal that α4βδ and α5βγ2 subunit-containing receptors mediate the majority of tonic inhibition in DGGC and CA1 hippocampal neurons, respectively (Caraiscos et al., 2004; Glykys et al., 2008). Similarly, neurosteroids have high affinity for the α6βδ-containing $GABA_A$ receptors, which are responsible for tonic inhibition within cerebellar granule cells (Hadley and Amin, 2007; Meera et al., 2011).

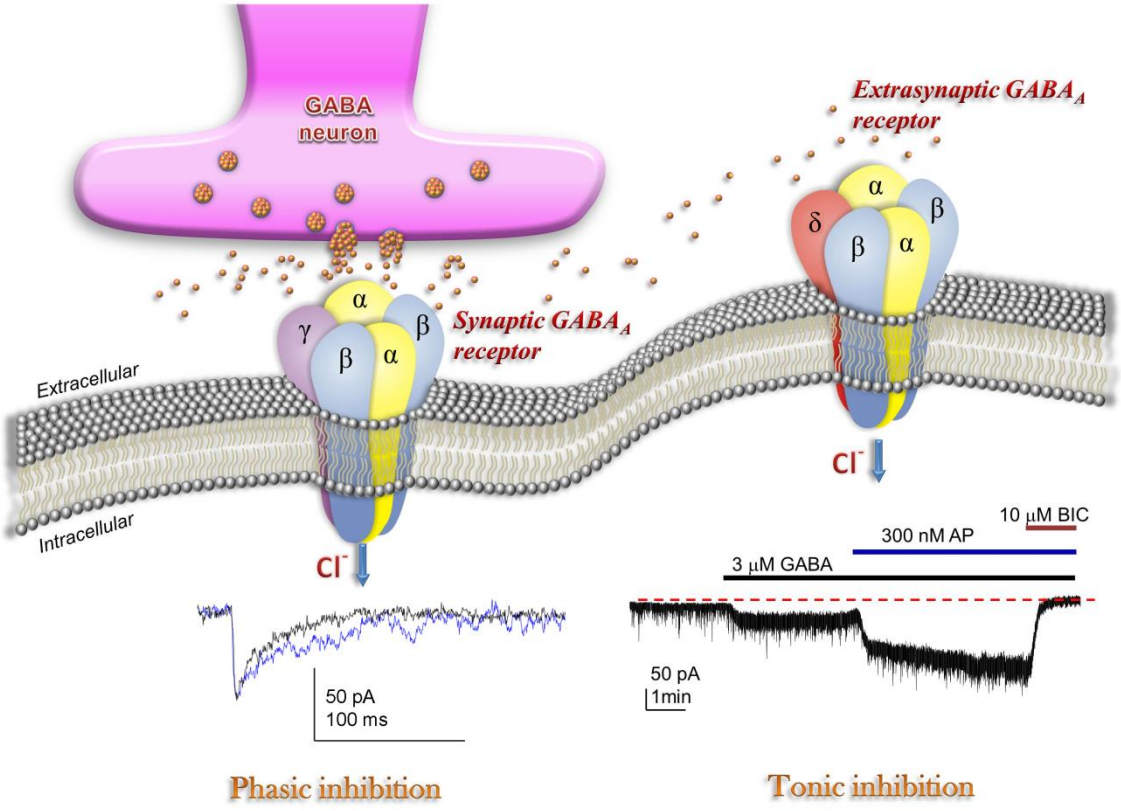


Figure 4. Neurosteroid modulation of synaptic and extrasynaptic GABA$_A$ receptors. Postsynaptic GABA$_A$ receptors are pentameric chloride channels composed of 2α:2β:1γ subunits and mediate the phasic GABAergic inhibition. Extrasynaptic GABA$_A$ receptors are pentamers composed of 2α:2β:δ subunits and primarily contribute to tonic inhibition in the dentate gyrus, thalamus, and cerebellum. Neurosteroids activate both synaptic and extrasynaptic receptors and enhance the phasic and tonic inhibition, thereby promoting maximal net inhibtion. The trace illustrating phasic inhibition depicts an IPSC produced by endogenous GABA vesicle release (black) and in the presence of 300 nM allopregnanolone (blue). Neurosteroids enhance IPSCs by prolonging the deactivation/decay kinetics, resulting in a longer mean channel open time. The trace illustrating tonic inhibition shows continuous, non-desensitizing chloride conductance activated by GABA (black bar) that was further potentiated by application of AP (blue bar). Bicuculline (BIC, red bar) blocks both phasic and tonic portions of GABAergic current.

Table 1. $GABA_A$ receptor pharmacological efficacy and potency

Drug Interaction	Efficacy	Potency	References*
AP and $\alpha_x\beta_1\gamma_2$	$\alpha_4 > \alpha_1 > \alpha_6 > \alpha_5 > \alpha_3 > \alpha_2$	$\alpha_3 > \alpha_1 > \alpha_2 > \alpha_6 > \alpha_5 > \alpha_4$	Belelli et al., 2002; Mortensen et al., 2012
AP and $\alpha_x\beta_{2/3}\delta$	$\alpha_6 > \alpha_4$	$\alpha_4 > \alpha_6$	Brown et al., 2002; Pillai et al., 2004; Mortensen et al., 2012
THIP	$\alpha_6\beta_3\gamma_2 > \alpha_1\beta_{2/3}\gamma_2 > \alpha_4\beta_3\gamma_2 > \alpha_4\beta_3\delta > \alpha_6\beta_3\delta$	$\alpha_6\beta_3\delta > \alpha_4\beta_3\delta > \alpha_6\beta_3\gamma_2 > \alpha_4\beta_3\gamma_2 > \alpha_1\beta_{2/3}\gamma_2$	Saarelainen et al., 2008; Mortensen et al., 2012; Meera et al., 2011
DS1 (δ-selective compound 1)	$\alpha_4\beta_3\delta > \alpha_4\beta_3\gamma_2 > \alpha_1\beta_3\gamma_2$	$\alpha_4\beta_3\delta > \alpha_4\beta_3\gamma_2 > \alpha_1\beta_3\gamma_2$	Wafford et al., 2009
DS2 (δ-selective compound 2)	$\alpha_4\beta_{2/3}\delta \approx \alpha_6\beta_2\delta >> \alpha_4\beta_3\gamma_2 \approx \alpha_1\beta_3\gamma_2$	$\alpha_4\beta_{2/3}\delta \approx \alpha_6\beta_2\delta >> \alpha_4\beta_3\gamma_2 \approx \alpha_1\beta_3\gamma_2$	Wafford et al., 2009; Jensen et al., 2013

*All data derived from heterologous expression systems in *Xenopus* oocytes or HEK293 cells.

Allopregnanolone-like neurosteroids are powerful anticonvulsants. Exogenously administered neurosteroids exhibit broad-spectrum anticonvulsant effects in diverse rodent seizure models (Reddy, 2010). Neurosteroids display rapid anticonvulsant activity and provide protection within minutes. Neurosteroids protect against seizures induced by $GABA_A$ receptor antagonists, including pentylenetetrazol and bicuculline, and are effective against pilocarpine-induced limbic seizures and seizures in kindled animals (Kokate et al., 1994; Belelli et al., 1989; Frye, 1995; Wieland et al., 1995; Reddy, 2004a,b; Reddy et al., 2010; Reddy and Rogawski 2010; Reddy and Kuruba, 2013). Like other GABAergic agents, neurosteroids are inactive or only weakly active against seizures elicited by maximal electroshock. Neurosteroids are highly active in the 6-Hz model, a paradigm in which limbic-like siezures are induced by electrical stimulation of lower frequency and longer duration than in maximal electroshock test (Kaminski et al., 2005, 2006). Neurosteroids are also highly effective in suppressing seizures due to withdrawal of $GABA_A$ receptor modulator drugs including neurosteroids, benzodiazepines, and alcohol, which may act in part through $GABA_A$ receptors, and cocaine, which does not (Tsuda et al., 1997; Devaud et al., 1996; Reddy and Rogawski, 2000a, 2001; Reddy et al., 2012). In contrast to benzodiazepines, where utility in the chronic treatment of epilepsy is limited by reductions in efficacy over time, anticonvulant tolerance is not obtained with neurosteroids (Kokate et al., 1998; Reddy and Rogawski, 2000b). Thus, neurosteroids have the potential to be used in the chronic treatment of epilepsy, and this has been evident in clinical trials (Nohria et al., 2010).

I.5 $GABA_A$ Receptor Pharmacology

Each $GABA_A$ receptor has two α-β subunit interfaces. GABA binds to the α-β interface to allow channel opening such that two GABA molecules may gate the receptor. The two interfaces differ in local environment based on the adjacent subunits (γ and β or α and γ) and therefore display some differences in agonist preferences (Baumann et al., 2003). Muscimol is a potent agonist for the GABA binding site and is often used to quantify pharmacology regarding allosteric binding of $GABA_A$ receptors by other agents. 4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridine-3-ol (gaboxadol, THIP) is a hypnotic agent and a partial agonist for $GABA_A$ receptors with a higher selectivity for δ-containing receptors (Ebert et al., 1997; Wafford and Ebert, 2006; Herd and Belelli, 2007; Herd et al., 2008), but displays different degrees of agonist binding that is dependent on the α subunit isoform included (Stórustovu and Ebert, 2003). The involvement of the δ-subunit in $GABA_A$ receptors confers a high THIP potency (Meera et al., 2011). GABA and

THIP have higher potency on α4β3δ than α4β3γ2L recombinant receptors expressed in *Xenopus* oocytes (You and Dunn, 2007). Furthermore, THIP has a higher efficacy than GABA for δ-containing receptors, but THIP has lower efficacy than GABA for γ2-containing receptors (Stórustovu and Ebert, 2006; Saarelainen et al., 2008). As potent receptor ligands, THIP and muscimol exhibit different degrees of agonist response in interaction with extrasynaptic function with high potency at α4/α6,β3δ receptors (Brown et al., 2002). High concentrations of THIP on α4β3δ receptors allow for longer and more frequent channel openings, while muscimol binding reduces the desensitization of the receptor (Mortensen et al., 2010). In comparison of α4β2δ and α4β3δ receptors, there does not seem to be a significant difference in the potency of GABA binding, signifying a less imperative role for β subunit variety dictating agonist binding properties (Mortensen et al., 2012).

Extracellular concentration of GABA has been predicted to be 0.4 μM at resting membrane potential (-80 mV) and 3.1 μM during depolarization (+20 mV) (Attwell et al., 1993). Extracellular GABA in the rat hippocampus has been measured to be between 0.8 and 2.9 μM using microdialysis (Lerma et al., 1986). Extracellular concentrations of extrasynaptic GABA have been estimated to be approximately 160 nM in experimental slice preparations (Santhakumar et al., 2006). GABA transporters likely have an important and dynamic role in controlling extracellular levels of GABA responsible for tonic inhibition (Richerson and Wu, 2003) (**Figure 5**). The prevalent perception is that surplus GABA spillover from vesicular synaptic release contributes to tonic current in neurons with δ-containing receptors (Hamann et al., 2002; Wei et al., 2003; Glykys and Mody, 2007).

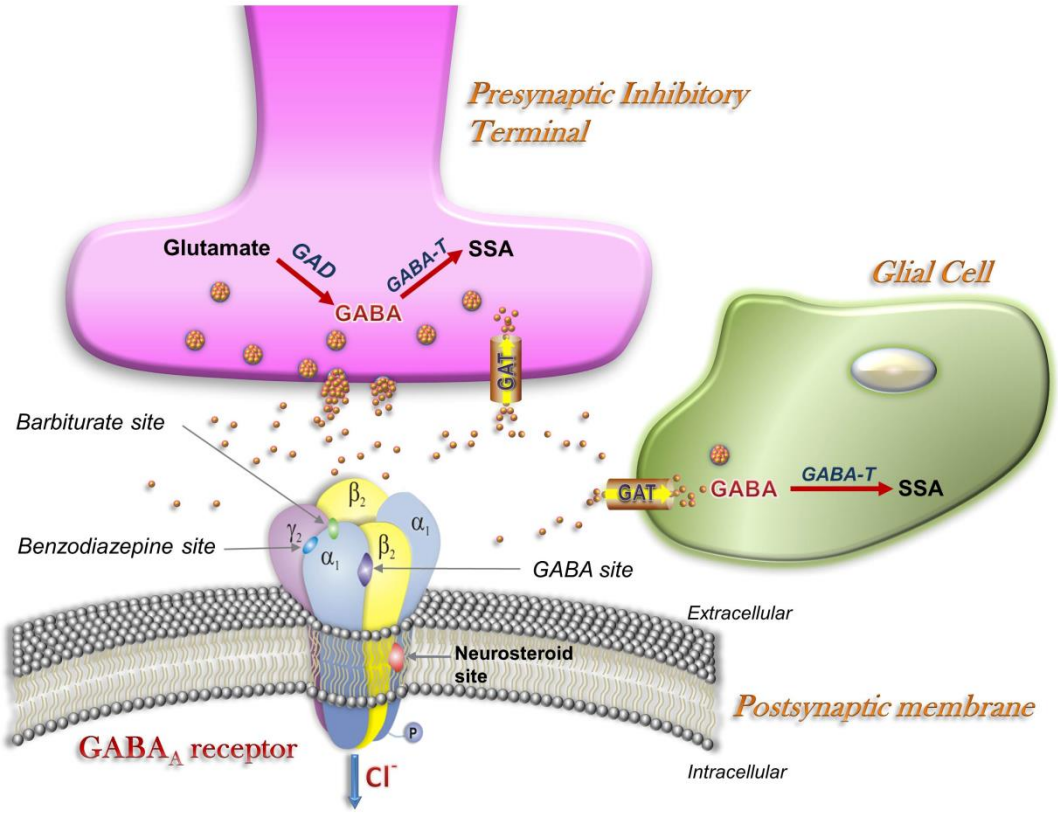


Figure 5. The GABAergic synapse and GABA reuptake. GABA, which is released from presynaptic vesicles, serves as the main fast inihbitory neurotransmitter in the brain by activation of postsynaptic $GABA_A$ receptors. A variety of chemical compounds are capable of binding to $GABA_A$ receptors to modulate its chloride channel function. Apart from GABA agonist sites, the receptor has specific sites for benzodiazepines, barbiturates, and neurosteroids, which are allosteric sites of modulation. GABA transporters in neurons (GAT-1) and glia (GAT-2/3) remove synaptically released GABA, thereby limiting or terminating its inhibitory action. Reuptake into terminals permits immediate recycling by vesicular uptake, whereas reuptake into astrocytes leads to metabolism via GABA-transaminase (GABA-T) and succinic semialdehyde (SSA) and glutamate. Binding of neurosteroids to their binding site(s) enhances the effect of GABA by increasing the frequency and duration of channel opening.

While there have been early attempts to determine binding patterns for neurosteroids, the true ligand-binding properties for neurosteroid-$GABA_A$ receptor interaction remain unclear. Neurosteroids have distinguishable binding sites from benzodiazepines, which act only on $GABA_A$ receptors containing γ subunits and those that do not have α4 or α6 subunits. Neurosteroids have been shown to possess a different binding site from barbiturates, though they share various similarities in receptor-mediated functions. The lipophilic properties of neurosteroids influence potency and affinity for $GABA_A$ receptors (Chisari et al., 2009). Molecular evidence shows that a C3,α-hydroxyl group and a C20 position ketone group on pregnane neurosteroids or a C17 ketone on androstane neurosteroids are essential for binding affinity (Mitchell et al., 2008). This suggests specificity in a binding pocket and interaction with certain residues yet to be fully clarified. Although the exact location of binding has not been mapped, it has been proposed that there are two distinct sites for neurosteroids that act as positive modulators: one for allosteric enhancement of GABA and another for direct activation of the receptor. There is also a negative modulatory site for sulfated neurosteroids involving the M2 region of the α subunit (Akk et al., 2001; Lambert et al., 2003; Hosie et al., 2006, 2007).

Using site-directed mutagenesis, it has been shown that a highly conserved glutamine at position 241 in the M1 domain (toward the intracellular side) of the α subunit plays a key role in neurosteoid modulation of GABA responses and is believed to contribute to the binding site for modulation (Hosie et al., 2009). Additional nearby residues in the M4 domain of the same α subunit (tyrosine 410 and asparagine 407, toward the extracellular side) have also been proposed to contribute to binding. Other investigators have found that mutations in serine 240 and tryptophan 245 of the α subunit interfere with neurosteroid potentiation. Multiple studies with structurally diverse steroids have led to the conclusion that the steroid binding pocket on the α subunit is more correctly viewed as a hydrophobic surface that can accommodate steroid molecules of different structures (Akk et al., 2009). Direct activation of the receptor, in contrast, has been proposed to be due to steroid binding at a site on the interface between β and α subunits formed by a threonine at position 236 in the α subunit and a tyrosine at position 284 in the β subunit (Hosie et al., 2007). However, more recent models of the $GABA_A$ receptor have questioned whether these residues reside at the β-α subunit interface. A photo-incorporable analog of the anesthetic etomidate appears to bind at the interface, but binding of this ligand is not competitively inhibited by neurosteroids (Li et al,. 2009). The β subunit has been less

prominently studied than α subunit in regard to neurosteroid binding. Early reports suggested that the β subunit does not appear to possess an influential role in neurosteroid interaction with $GABA_A$ receptors (Lambert et al,. 2003). However, β2 subunits are preferentially assembled in extrasynaptic channels with high neurosteroid affinity and β2 deficiency reduces tonic inhibition, while β3 subunit-containing receptors are mostly synaptic, based on dentate gyrus studies (Herd et al., 2008). Manipulation of the M1 domain of the β2 subunit has an effect on the spontaneous activity of extrasynaptic receptors (Baker et al., 2010). The extracellular domain loop 9 of the β2 subunit has been shown to steady the closed-state gate of the α1 β2γ2S synaptic $GABA_A$ receptors, and this has indirect consequences on receptor efficacy in GABA activation and the allosteric modulation by propofol and pregnanolone (Williams et al., 2010).

A recent report identified a novel β3 subunit transmembrane domain involved in neurosteroid binding (Chen et al., 2012). While β isoforms have been found to influence tonic current (Herd et al., 2008), it is still unclear if they significantly affect neurosteroid modulation. The δ subunit is integral to the high sensitivity of neurosteroid-receptor interaction (Brown et al., 2002). The δ subunit is not germane to the actual neurosteroid binding site for opening of the channel but rather is involved in the transduction of the GABA-gated response subsequent to initial binding (Hosie et al., 2009). Channels of α4β2δ do not differ in GABA potency from channel pentamers composed of α4β2 (Mortensen et al., 2012). Moreover, recombinant receptors modified to contain a single functional GABA agonist site and/or a single proposed allosteric site are adequate in elicting potentiation by AP, due to the potent sensitivity to steroids (Bracamontes et al., 2011). Physiological receptors have much more diverse kinetics relative to direct and allosteric neurosteroid binding, and the details surrounding those binding targets remain unclear. However, the δ subunit possesses an intrinsic property to increase channel opening probability in potentiating current. Therefore, the α-β interface without δ may permit neurosteroid affinity, but the δ incorporation provides a potentiating shift for longer channel opening and gating of current (Bianchi et al., 2002). Mutation in the M2 region of δ-containing receptors decreases the potency of the positive allosteric modulators THDOC and tracazolate, which typically potentiate $GABA_A$ receptors at low-efficacy (Zheleznova et al., 2008). In addition, the S238-V264 domain of the δ subunit (including M1 and the intracellular loop connecting to M2) has been identified to influence the high level of agonist sensitivity to α4β3δ receptors apparent in both measures of efficacy and potency (You and Dunn, 2007). Binding of receptor is not likely to occur at theses δ

subunit domains by either GABA or neurosteroid, but these domains nevertheless have profound tranduction effects on channel gating.

Despite the fact that neurosteroid function diverges from the classical steroid role of diffusion across the membrane to act through nuclear receptors, the lipophilic structure of neurosteroids permits their movement in cells and the plasma membrane similarly to other steroids. Potentiation of $GABA_A$ receptor channels occurs upon application of AP to the inner leaflet of transfected membrane in an inside-out patch configuration (Akk et al., 2005). In conjunction with other data showing the ability of neurosteroids to reach receptors through lateral diffusion or from intracellular paths, this substantiates that neurosteroids are able to modulate current through routes other than extracellular access (Akk et al., 2009). Access via a hydrophobic pocket of the receptor within the membrane bilayer therefore remains a feasible biochemical explanation for mechanism of neurosteroid binding and affinity.

I.6 Electrophysiological Basis of GABAergic Current

Pharmacological modulation of $GABA_A$ receptors is measured by inhibitory conductance and channel kinetics, which provide important insights to the differences between phasic and tonic receptors. Neurons with high expression levels of extrasynaptic receptors involved in tonic current have distinct, unique properties from neurons with primarily phasic input (Stell and Mody, 2002). Single channel patches of neuronal membrane $GABA_A$ receptors possess channel gating properties of sensitivity and comparatively fast recovery of inward current. Synaptic inhibition is characterized by biphasic (fast followed by slow) desensitization and delayed deactivation of the channel's inward current in response to GABA, and this is typified by the αβγ population of receptors which are prominent at the postsynaptic membrane. In contrast, extrasynaptic receptors are exemplified by a single phase of decay, more rapid deactivation, and a lesser degree of desensitization to GABA-induced current (Bianchi et al., 2001; Brown et al., 2002; Kaur et al., 2009). Specific residues of the δ subunit M1 domain and N terminus are necessary for the observed extrasynaptic desensitization kinetics, and mutations in the same region of γ2L result in increased fast deactivation (Bianchi et al., 2001). Endogenous neurosteroids modify neuronal $GABA_A$ receptor current by increasing the channel opening

duration, prolonging slow deactivation, and delayed recovery from desensitization (Zhu and Vicini, 1997; Wohlfarth et al., 2002).

Since extracellular GABA is responsible for setting the inhibitory tone, GABA transport and its maintenenance within brain have large bearing on the control of tonic current. Depletion or inhibition of GAT-1 raises levels of GABA, and this has been explored as a therapeutic target for anxiolytic, depressive, and epileptic disorders (Kristensen et al., 2011). The GABA transporters may serve roles beyond recylcing GABA into the axon terminal for vesicular release. GABA transport and reuptake also displays a significant role in modulating the level of baseline tonic current for dendritic regions of the neuron. Furthermore, allosteric modulators of tonic current undergo regulation of receptor binding sensitivity due to GABA transporters (Fleming et al., 2011). Altered sodium electrochemical gradients may contribute to ambient extracellular GABA by reversing the direction of GAT-1 transport (Attwell et al., 1993; Wu et al., 2006, 2007). In addition, GABA synthesis itself is significant in extrasynaptic function, as lack of GAD65 reduces tonic inhibition (Walls et al., 2010).

$GABA_A$ receptor-mediated tonic currents function based on a mechanism of control derived from channel properties and other exogenous stimuli (McCartney et al., 2007; Ransom et al., 2010). Certain neuronal regions exhibit high degrees of tonic inhibition determined by channel opening kinetics, even though tonic inhibition is observed throughout the brain. Neurosteroids have been shown to selectively potentiate $GABA_A$ receptor inhibition in a fashion independent to subunit composition, as revealed by switching the polarity of efficacy in αβγ and αβδ populations (Bianchi and Macdonald, 2003). The intrinsic low-efficacy activity of physiological δ-containing receptors is exemplified by brief channel openings and low levels of desensitization. The low-efficacy characteristics enable neurosteroids to increase the mean channel open time in their potent modulation of receptors. Neurosteroids acting on α4β3δ receptors display a greater efficacy than on α4β3γ2 receptors (Brown et al., 2002). Mutation of residue L286S of the δ subunit modifies $GABA_A$ receptors to higher efficacy and channel characterisitics similar to that of γ-containing receptors (Zheleznova et al., 2008). Furthermore, differences in gating are expressed through altering α subunit isoforms. With a saturating concentration of GABA (1 mM), α1β3δ channels desensitize slowly, deactivate rapidly, and exhibit peak-amplitude voltage-dependence. In contrast, α6β3δ channels have voltage-dependent

desensitization and slower deactivation but do not show rectification (Bianchi et al., 2002). Therefore, it is not necessarily the subunit composition per se that confers the neurosteroid capacity to modulate tonic current. The channel efficacy properties of the subunit phamacophore confer the potent affinity for endogenous neurosteroids. As neurosteroids interact with extrasynaptic $GABA_A$ receptors, the high-affinity binding increases gating efficacy and is manifested as potent inhibitory function.

Tonic current is typically quantified through whole-cell, voltage-clamp recording of a brain slice preparation. Inhibitory activity is represented in terms of conductance which is normalized by cell membrane capacitance as current density (pA/pF). This allows for control of variability based on the membrane surface area contributing to overall capacitance. Baseline currents are initially measured and averaged form epoch increments to signify GABAergic-mediated current. Saturating concentrations of $GABA_A$ receptor antagonist bicuculline or gabazine (SR-95531) are then applied to the recording chamber to identify the loss of current due to blocking of the ion channel. Extrasynaptic channels have been previously classified as relatively insensitive or resistant to gabazine due to inability to inhibit $GABA_A$ receptors that are active in the absence of GABA (Bai et al., 2001; Yeung et al., 2003; McCartney et al., 2007). However, these data were based on pyramidal cell tonic currents, which display lower levels of chloride conductance than δ-containing DGGCs, and gabazine antagonism is largely concentration dependent (Stell and Mody, 2002; Stell et al., 2003; Houston et al., 2012). Upon pharmacological antagonism, the change in current from active to blocked condition is quantified to calculate the tonic conductance for the cell. GABA transporter and uptake blockers, such as the GAT-1 inhibitor NO-711, may maintain or increase exogenous levels of GABA and may even enhance tonic inhibition without altering phasic current (Nusser and Mody, 2002).

I.7 Functional Characteristics of δ-containing Receptors

In addition to distinctive pharmacological affinities, δ-containing $GABA_A$ receptors also possess unique current-gating properties that play a role in control of neuronal excitability. Knockout mouse models allow for disambiguation of properties influencing GABAergic current. At the time of the creation of the first δ knockout (δKO) mouse in 1999, little was known about the extent of neurosteroid interaction with δ-containing $GABA_A$ receptors. The construction and classification of the δKO was seminal in discerning the true activity and physiology of

neurosteroids at $GABA_A$ receptors. The initial δKO demonstrated that removal of δ subunit substantially diminishes neuronal sensitivity to neurosteroids (Mihalek et al., 1999). Alfaxolone potentiation is lessened in δKOs, accompanying reduction in hippocampal expression of α4 subunit (Spigelman et al., 2003). The hypnotic activity of THIP is reduced when administered to δKO mice, signifying high affinity for THIP at δ-containing receptors (Boehm et al., 2006; Meera et al., 2011). The δKO mice display lower behavioral impairment and reduced binding sensitivity to muscimol, the high-affinity $GABA_A$ receptor agonist (Chandra et al., 2010). The δ-specific profiles confirm shifts in channel gating due to endogenous neurosteroid modulation (Wohlfarth et al., 2002), which is attenuated in the δ-deficient model (Stell et al., 2003).

Knockout constructs and electrophysiology have revealed that the dentate gyrus has high levels of $GABA_A$ receptor expression (Pirker et al., 2000; Peng et al., 2002). α4βδ extrasynaptic receptors comprise a majority of the tonic current observed in DGGCs (Sun et al., 2004; Chandra et al., 2006; Herd et al., 2008). Receptors containing α5βγ2 are responsible for a minor residual tonic current in DGGCs of δKO animals, but represent a majority of tonic current in CA1 pyramidal neurons (Glykys et al., 2008). In addition, dentate gyrus molecular layer interneurons (MLI) are composed of α1βδ receptors (Glykys et al., 2007; Wei et al., 2013). Concomitant with δKO characteristics, α4 knockout mice display decreased GABAergic potentiation by synthetic alfaxalone in DGGCs (Liang et al., 2008). This mirrors physiological deficits of neurosteroid potentation in mice lacking δ subunit and provides evidence for steroidal selectivity.

The expressional differences of δ subunit between DGGCs and CA1 pyramidal cells may reveal an important clue to understanding extrasynaptic receptor activity and function. The estrous cycle serves as a useful model because tonic inhibition changes are dependent on cell-type and receptor-type specificity. Due to changes in steroid hormone levels during the estrous cycle, neurosteroid sensitivity is modulated differently in DGGC and CA1 in conjunciton with $GABA_A$ receptor plasticity. DGGC tonic conductance is increased in diestrus, but no cycle-dependent changes in conductance are observed in CA1 pyramidal cells (Maguire et al., 2005; Wu et al., 2013). Whole-cell, GABAergic currents from native, adult rat DGGCs are highly sensitive to positive modulation by AP and negative modulation by PS (Mtchedlishvili et al., 2003). Tonic current in DGGCs is mediated by δ-containing receptors and is sensitive to neurosteroid;

however, tonic current in CA1 pyramidal cells is not mediated by δ-containing $GABA_A$ receptors (Stell et al., 2003; Glykys et al., 2008).

Mice deficient in δ subunit also exhibit pathologically relevant changes in conjuntion with reduced neurosteroid sensitivity. The GABA antagonist pentylenetetrazol elicits a significantly greater number of clonic seizures in δKO mice compared to control animals (Mihalek et al., 1999; Spigelman et al., 2002). This indicates a higher degree of acute seizure susceptibility marked by loss of δ-mediated inhibition. Hippocampus kindling has not yet been implemented within the δKO model to further typify limbic epileptiform activity. Furthermore, DGGCs from δKOs demonstrate faster current decay rates of mIPSCs and evoked IPSPs, but there have been no observed changes in frequency, rise time, or amplitude (Spigelman et al., 2002). DGGCs from δKO mice have highly attenuated tonic current, and α5/δ double knockout mice effectively lack any tonic inhibition within hippocampal neurons (Stell et al., 2003; Glykys et al., 2008). CA1 pyramidal cells possess $GABA_A$ receptors with a lower amount of α4βδ compared to DGGCs (Peng et al., 2004), and the relatively low tonic conductances are not changed between wild-type and δKO mice (Stell et al., 2003; Maguire et al., 2005). The δ-containing receptors have high sensitivity to Zn^{2+}, and wild-type mice DGGCs experience reduced spontaneous IPSC decay time in response to $ZnCl_2$ application. In contrast, spontaneous IPSC decay time is not affected by $ZnCl_2$ in δKO neurons (Saxena and Macdonald, 1996; Wei et al., 2003). These findings convey a reduced ability of extrasynaptic, tonic current to control excitability, owing to an important niche role for δ-containing receptors. Two missense mutations have been identified from generalized epilepsy febrile seizure plus human patients (Dibbens et al., 2004). In addition, a homozygous δ subunit mutation was found in a case of juvenile myoclonic epilepsy. In a recombinant system, α4β2δ receptors with similar mutations were examined regarding their channel gating characteristics (Feng et al., 2006). These receptor variants display decreased GABA current amplitudes and decreased single channel mean open times. These reports suggest that heritable $GABA_A$ receptor δ subunit gene (*Gabrd*) variations can produce a higher susceptibility to excitation and epileptic seizures.

I.8 Subunit Expressional Plasticity

Subunit expression differences within $GABA_A$ receptors are reflected by conditional changes such as development, stress, pregnancy, partruition, and ovarian/hormonal cycling. These plastic

changes are contextually relevant to further elucidating the function of neurosteroids on GABAergic neurons (Smith et al., 2007; Belelli et al., 2009). Pathophysiology studies corroborate evidence that irregular, dysfunctional states of neural networks like epilepsy involve alterations in subunit expression. Many studies report compensational changes of subunit expression due to genetic, functional, or pharmacological changes to the control of GABAergic inhibition (Peng et al., 2002; Liang et al., 2008; Gangisetty and Reddy, 2010; Uusi-Oukari and Korpi, 2010; Suryanarayanan et al., 2011; Kuver et al., 2012). Evidence for neurosteroid-dependent modulation of receptor plasticity is beginning to emerge. Therefore, in conditions where there is a substantial change in endogenous neurosteroid levels, receptor subunit reconfigurations could occur.

Initial electrophysiology studies on DGGCs in δKO mice reported that there was no overarching change in synaptic inhibition, but an observed faster rate of decay for mIPSCs, suggesting that structural receptor modifications might occur (Mihalek et al., 1999). Molecular anaysis of mRNA from δKOs reveals a localized shift in which expression of γ2 subunit increases and α4 subunit expression levels decrease in the forebrain, implicating functional compensation in response to the loss of δ (Korpi et al., 2002; Peng et al., 2002). Hippocampal $GABA_A$ receptor subunit protein levels of δKOs also demonstrate that with constitutive loss of δ subunit, α4 levels are diminished, coupled to observed decreases in inhibition (Spigelman et al., 2003). In α4 knockout mice, γ2 subunit levels increase within the hippocampus, further signifying a modulatory mechanism for balancing inhibition within this brain region (Liang et al., 2008). The compensational changes to other subunits has been a challenge to understanding the putative role of δ-subunit receptors. Increasing evidence suggests that δ-containing receptor function affects key aspects of hippocampal neurotransmission, however other compensatory mechanisms may persist within the null transgenic mice.

While receptor expression data from knockout mice show signs of compensatory adaptation, the regulatory processes involved in subunit assembly and incorporation of receptors into the membrane remain to be discovered. It is possible that neurons with high extrasynaptic function depend on endogenous neurosteroids to adjust baseline inhibition. These neurons cannot rely on synaptic GABAergic transmission for inhibitory set point control due to the transient availability of synaptic GABA and the considerable distance from the synapse. However, extrasynaptic

receptors are fine-tuned to allow modulation by secondary ligands that adjust channel gating. It appears that certain subunit isoforms are preferentially assembled together, and their location on neuronal membrane reflects the regulatory needs of the neuron (Bogdanov et al., 2006). Conditional changes that augment expressional regulation provide a larger understanding of functional maintenance of $GABA_A$ receptors and how neurosteroids influence this plasticity.

I.8.1 Development

Formation of neural networks in the hippocampus has provided insight into receptor plasticity in the developing brain. In CA1 pyramidal neurons of embryonic and postnatal rats, a form of tonic inhibition is observed even before synapses are formed (Demarque et al., 2002). Interestingly, this mechanism of inhibition precludes the requirement of transient, synaptic GABA release to be goverened by Ca^{2+} influx and GABA reuptake controlled by GAT-1. Tonic inhibition is also active at early developmental stages of the dentate gyrus and is important in regulating the excitation/inhibition balance in the maturing hippocampus (Holter et al., 2010). Within rat hippocampus, α4 and δ subunit proliferation of expression occurs late in postnatal development and into adulthood (Laurie et al., 1992). By the time adult age is reached, synaptic α and γ2 containing receptors are already highly expressed and established within the brain. Both α2 and α5 subunit expression increases in the mouse hippocampus in the initial postnatal months of brain development and reach and maintain peak levels during adulthood. The γ2 subunit expression declines in the aging brain (Yu et al., 2006). This study implies that gene expression of α2 and α5 is induced, and γ gene expression is down-regulated due to the large changes across neural development in multiple brain regions. In DGGCs, the $GABA_A$ receptor-mediated currents have been found to increase in sensitivity to AP as rats mature, wherein α1 expression also increases from birth to adulthood (Brooks-Kayal et al., 2001; Mtchedlishvili et al,. 2003). There are also developmental changes from α5 subunits α1, α2, and γ2 in DGGC, linked to high zinc sensitivity of receptors, which subsequently declines and becomes channel blocking as neurons mature (Galanopoulou, 2008ab). As the brain establishes the structural and chemical foundations critical for neuronal activities, neurosteroid synthesis and modulation of $GABA_A$ receptors likely play a role in shaping function.

I.8.2 Estrous cycle

Exploration into the fluctuations in steroid synthesis and circulation during the ovarian cycle has yielded critical information as to the function of neurosteroids on $GABA_A$ receptors. The ovarian cycle progresses with consequent endocrine changes in estradiol and progesterone levels. In the rodent estrous phase, progesterone levels are typically low and estrogen levels are high. During the diestrous phase, heightened levels of progesterone induce its metabolite, AP, to increase during luteal surges within the ovary and plasma (Reddy and Kulkarni, 1999; Reddy, 2009a). $GABA_A$ receptor subunit gene expression is affected by oscillations in the ovarian cycle due to this rise in neurosteroid concentration not only in peripheral tissue but also in the brain. This expressional response promotes gene activation and results in a change in amount of specific receptors that are functional on the neuronal membrane. During the diestrous phase, receptor protein levels of δ subunit increase in the hippocampus compared to the estrous phase, and this is coupled to significant increases in GABAergic tonic conductance in DGGCs (Maguire et al., 2005; Wu et al., 2013). In kindling studies, susceptibility to hippocampus epileptogenesis has been lower in mice during diestrus. Levels α4 do not change significantly between these two phases of the cycle. The mirrored compensation of specific subunit isoform up-regulation in response to neurosteroid deficit in the central nervous system implicates a substantial system of neuromodulatory control of GABAergic inhibition. Over the course of the cycle, neither progesterone receptor antagonist RU846 nor estrogen receptor antagonist tamoxifen affects regulation of $GABA_A$ receptors in the brain, suggesting neurosteroid-dependent plasticity (Maguire and Mody, 2007; Reddy et al., 2010; Wu et al., 2013). Control of $GABA_A$ receptor populations in puberty and into adulthood may be maintained by an overarching feedback mechanism involving the neuroendocrine axis balance of hormones. Therefore, developmental defects or pathophysiological onset of disorder within this system could greatly alter the excitability of the brain, such as in catamenial epilepsy, premenstrual syndrome, and migraine (Reddy, 2013a).

I.8.3 Neurosteroid treatment

Treatment of animals with steroid mimics subunit expression compensation and plasticity. Administration of preogesterone or pregnenolone selectively and substantially increases 3α,5α-reduced neurosteroid levels in both rodents and humans and can be measured in the plasma by

high throughput methods (Porcu et al., 2009). Gonadotropin treatment increases AP levels (Reddy et al., 2012). Progesterone and AP treatment can up-regulate α4 and δ in the hippocampus, documented in both in vitro and in vivo functional studies (Biggio et al., 2006; Uusi-Oukari and Korpi, 2010). Up-regulation of δ subunit in hippocampus after AP treatment is significant for channel kinetics regarding potentiation; both efficacy and potency to THIP increase following neurosteroid administration. This direct alteration of $GABA_A$ receptor plasticity reflects subunit targeting specificity in which δ is involved, but it also modifies the functionality of receptors where α4 and γ2 are coexpressed. In response to neurosteroid treatment, CA1 synaptic current decay time is reduced (Hsu et al., 2003). A brief 48-h treatment with AP highly increases the expression profiles of the α4 and δ composition and decreases expression of the typically synaptic α1 and γ2 subunits within rat CA1 (Shen et al., 2005). However, δ-containing receptors are not as functionally significant at CA1 as compared to the dentate gyrus subfield, where expressional and functional changes to $GABA_A$ receptors have a higher impact on network excitability. Neonatal exposure to estradiol in female rats decreases brain levels of progesterone and AP in addition to increasing the quantity of synaptic α1, α2, γ2 subunits with no change to α3, α4, α5, or δ subunits (Calza et al,. 2010). An increase in anxiolytic behavior results when diazepam is applied to estradiol-treated animals, but there is little change by AP. This estradiol-modulated, increased sensitivity exhibits subunit plasticity-directed specificity to allosteric ligands concerning broader $GABA_A$ receptor gating. These observations are clinical relevant during chronic neurosteroid therapy.

I.8.4 Neurosteroid withdrawal

Withdrawal from endogenous neurosteroids has striking impact on subunit plasticity within the hippocampus. Neurosteroid withdrawal (NSW), which occurs during the perimenstrual period, is linked to catamenial epilepsy and increased incidence of seizure susceptibility in women (Reddy et al., 2001; Reddy, 2013a, b). In rat hippocampal cultures, withdrawal following chronic exposure to either progesterone or AP causes a transient increase in δ subunit expression (Mostallino et al., 2006). Induced neurosteroid withdrawal following chronic doses of progesterone or AP in rodents has shown to increase the expression of α4 subunit approximately 3-fold (Smith et al., 1998b; Gangisetty and Reddy, 2009, 2010). Progesterone and neurosteroid withdrawal models exhibit an up-regulation in α4 and δ subunits with effects on current, pharmacology, and seizure susceptibility (Sundstrom-Poromaa et al., 2002; Reddy et al., 2012).

Due to NSW-dependent increases of α4 and δ subunits, fully kindled mice experience enhanced sensitivity to the antiseizure effects of AP (Reddy et al., 2012). There is no observed compensational shift in α1, α2, β2, γ2 subunits in response to withdrawal. This may represent a divergent mechanism of expression for synaptic and extrasynaptic receptors.

Increase in seizure activity and excitation of the brain has differential effects on $GABA_A$ receptor physiology of CA1 and DGGCs (Gibbs et al., 1997). Progesterone withdrawal has been linked to α4-dependent increase in CA1 pyramidal neuron excitability (Hsu and Smith, 2003). In CA1 subfield, GABAergic inhibition is primarily mediated by other subunits (Caraiscos et al., 2004; Prenosil et al., 2006). The α4 subunit is more prevalent in dentate gyrus than in the CA1 region (Pirker et al., 2000). Alterations to receptor function within the dentate gyrus have severe effects on the control of epileptiform spread of hyperactivity (Coulter and Carlson, 2007). Extrasynaptic profiles of δ-containing receptors reflect considerable plasticity to neurosteroid withdrawal that influences their channel opening probability (Bianchi and Macdonald, 2003). Phasic and tonic $GABA_A$ receptors may rely on disparate mechanisms that control expression based on neuronal requirements to maintain network inhibition. Due to the high neurosteroid sensitivity to $GABA_A$ receptors mediating tonic current within the dentate gyrus, it is probable that neurosteroids drive expressional changes to regulate baseline inhibition.

I.8.5 Epilepsy

Epilepsy is a neurological disease characterized by recurrent seizures resultant from aberrant hyperexcitability within the brain. While there are many types of epileptic seizures, certain types may be studied to elucidate the nature of the disease and formulate appropriate treatments in control of seizures. Receptor plasticity has been reported in several models of epilepsy, implicated to play a role in the molecular changes that promote seizure susceptibility. Alterations to the function of $GABA_A$ receptors through a pilocarpine-induced model of temporal lobe epilepsy (TLE) reveal that CA1 neurons from epileptic animals undergo a reduced maximal efficacy (decrease of 45%) to GABA without a change in potency and increased sensitivity to zinc blockade of current (Gibbs et al., 1997). Epileptiform bursting activity promotes cellular internalization of $GABA_A$ receptors and has consequent effects on synaptic GABA transmission in reducting inhibition (Goodkin et al., 2005). Both mature and newborn dentate cells undergo reductions in spine density and number in response to induced status epilepticus (Santos et al.,

2011). Hippocampal neurons possess varying $GABA_A$ receptor compositional changes in response to hyperexcitability of the brain. The foundation for greater seizure susceptibility is maintained by the larger decrease in available neurosteroid in the brain, altered receptor expression level on the neuronal surface and reduced overall GABAergic inhibition.

Catamenial epilepsy is characterized by increased seizure frequency due to menstrual cycle-related hormonal fluctuations (Reddy, 2007, 2009a; Reddy et al., 2012). Deficits in cyclical fluctuations in steroid hormones and receptor plasticity play a central role this condition. The seizures produced by the disease are believed to result from a cyclic, temporal deficit of circulating neurosteroid, which in turn increases seizure susceptibility in conjunction with a dearth of GABAergic inhibitory control over excitability (Reddy 2004ab; 2009a; 2013ab). The δ-containing receptors are crucial mediators of tonic inhibition and limbic epileptogenesis and are regulated within the estrous cycle (Reddy et al., 2012; Wu et al., 2013). In women with perimenstrual catamenial epilepsy, overall THDOC levels and DHEAS/cortisol ratio are reduced, but the pregnane steroids progesterone, pregnenolone, and AP are not different compared to control groups (Tuveri et al,. 2008). However, in the perimenstrual part of the cycle, the luteinizing surge produces a rapid flux in progesteone and its metabolites that afterward sharply decrease (Reddy, 2009a). Partruition is also associated with a substantial decrease in progesterone and AP levels, and there is a postpartum increase in α4 and γ2 subunit expression, but decrease in δ within the hippocampus (Sanna et al., 2009). Thus, epilepsy may cause a different compensational mechanism of $GABA_A$ receptor plasticity within the brain compared to the physiological condition of progesterone withdrawal in order to allow for parturition to proceed.

Treatment with neurosteroids has shown to reduce seizure activity in several epilepsy models (Lawrence et al., 2010; Reddy and Jian, 2010; Reddy et al., 2010). Noda model epileptic rats have decreased δ subunit expression in DGGCs along with decreased tonic current, smaller tonic response to AP (Pandit et al., 2013). Furthermore, in Noda rat slices, AP prolonged synaptic receptor decay in DGGC similarly to the normal Wistar strain counterparts. Pilot studies from our lab show that AP retains its antiepiletic efficacy in this animal model of TLE and is successful in suppressing the occurrence of spontaneous seizures in chronically epileptic rats (Reddy et al., 2005). Better discernment of the role of neurosteroid-receptor interactions in the

context of epilepsy necessitates that in vitro studies are corroborated with in vivo studies that typify the whole network response to $GABA_A$ modulation.

The physiological, intrinsic low excitability of the dentate gyrus has an important role in the filtering of excitatory activity from the entorhinal cortex and regulating propagation of current that passes through to other hippocampal neuronal tissue (Heinemann et al., 1992; Lothman et al., 1992; Coulter and Carlson, 2007). DGGCs from TLE rats have increased synaptic efficacy, exemplified by increase in number of postsynaptic channels, but not channel conductance (Otis et al., 1994). However, these changes to miniature IPSC amplitude do not take into account possible alterations in extrasynaptic $GABA_A$ receptors. Kainate treatment to induce seizures decreases frequency of miniature IPSCs, but does not change the frequency of action potential-dependent, spontaneous IPSCs (Shao and Dudek, 2005). While IPSC recordings do not provide a comprehensive view of how epilepsy modifies GABAergic inhibition, these findings denote compensatory effects within the hippocampus to maintain inhibitory current. Observed reductions in phasic inhibition in epileptic animals agree with evidence that γ2-containing receptors may increasingly shift to extrasynaptic sites and assemble with α4 subunits. DGGC receptor plasticity in epileptic mice is reported to have static α4 expression, δ down-regulation, and γ2 increase in dendritic areas of neurons (Zhang et al., 2007).

In contrast, epileptiform DGGCs have been found to display increased inhibition of mIPSCs and altered $GABA_A$ receptor sensitivity to zinc and benzodiazepine pharmacology, also associated with increased membrane receptors (Cohen et al., 2003). There are various contrasting findings regarding changes in dentate gyrus inhibition that could be interpreted by the location of the synaptic inhibition recording (Zhang et al., 2007). Pharmacological and electrophysiological evidence of pilocarpine-induced status epilepticus also suggests enhanced tonic, GABAergic inhibition, largely modulated by L-655,708-sensitive α5-containing receptors in lieu of δ-containing receptors (Zhan and Nadler, 2009). There is still considerable uncertainty regarding how receptor physiology influences GABAergic inhibition in epileptic animals; however, the rodent model of TLE provides indication of compensatory shifts in subunit composition that influences tonic current (Mody, 2012). Nevertheless, pharmacological shifts in receptor subunit sensitivity are concomitant with changes in receptor subunit composition.

CHAPTER II
AIMS AND OBJECTIVES

The main objective of this dissertation research is to understand the role of δ-subunit-containing $GABA_A$ receptors in the hippocampus dentate gyrus in mediation of tonic inhibition and epileptogenesis using a combination of electrophysiological, behavioral, and pharamcological techniques. Transgenic mouse models including germline δ-subunit knockout mice (δKO) were used to elucidate the behavioral and physiologic sensitivity to neurosteroids as targets for extrasynaptic $GABA_A$ receptors within the hippocampus as a key brain structure for the pathophysiology of epilepsy. The research is organized into three specific aims.

II.1 Specific Aim 1

The first specific aim investigates the plasticity and function of δ-containing $GABA_A$ receptors in the hippocampus in a perimenstrual-like model of neurosteroid withdrawal.

Neurosteroids play a key role in the pathophysiology of catamenial epilepsy, a menstrual-cycle related disorder characterized by seizures that cluster most often during the perimenstrual or periovulatory period, when progesterone levels are low (Herzog and Frye, 2003; Herzog et al., 2004; 2011; Reddy et al., 2012). Presently there is no approved drug therapy for catamential epilepsy. Progesterone is a precursor for the synthesis of neurosteroids such as allopregnanolone in the brain (Reddy et al., 2004; Tuveri et al., 2008). AP and related neurosteroids have anticonvulsant properties and protect against seizures. Although the exact cause of catamenial epilepsy is poorly understood, there is growing evidence suggesting perimenstrual neurosteroid withdrawal (NSW) may be a key triggering factor for catamenial seizures (Smith et al., 1998ab; Reddy et al., 2001, 2012; Reddy, 2009ab; Gangisetty and Reddy, 2010; Pack et al., 2011). We previously developed an animal model of catamenial epilepsy (Reddy et al., 2001; 2012; Reddy and Zeng, 2007). In this rodent model, there is a marked reduction in the antiseizure potency of benzodiazepines and valproate, which is consistent with clinical evidence on resistance of catamenial seziures to conventional antieplielptic drugs (Reddy and Rogawski, 2001; Reddy et al., 2012). However, neurosteoids including AP and synthetic analogs such as ganaxolone have enhanced activity in the catamenial epilepsy model (Reddy and Rogawski, 2000; 2001; Reddy et

al., 2012). Neurosteroid replacement therapy for prevention of catamenial seizures has been previously proposed (Reddy and Rogawski, 2009; Reddy, 2013). However, the molecular mechanisms underlying enhanced anticonvulsant activity of neurosteroids in catamenial epilepsy remain unclear.

There is indication that steroid hormone fluctuations affect δ-subunit plasticity (Smith and Gong, 2005; Reddy et al., 2012). Therefore, we hypothesized that the enhanced potency of neurosteroids in catamenial epilepsy may be due to a relative increase in the expression of extrasynaptic, δ-containing $GABA_A$ receptors in the hippocampus. In the first specific aim, we tested this hypothesis by investigating the NSW-induced changes in $GABA_A$ receptor δ-subunit expression and tonic inhibition in the dentate gyrus in a perimenstrual mouse model.

II.2 Specific Aim 2

The second specific aim investigates the specific structure-activity relationship of neurosteroid for modulation of tonic inhibition in mice in wildtype condition as well as germline deletion of δ-subunit-containing $GABA_A$ receptors.

Tonic currents were investigated as a basis for the structure-specific neurosteroid modulation on extrasynaptic receptors within the dentate gyrus. In case of synaptic receptors, neurosteroids bind to $GABA_A$ receptors via a hydrophobic pocket within the neuronal plasma membrane. There are two discrete binding sites, including an allosteric site in the α-subunit transmembrane domain and a site of direct activation at the α-β subunit interface (Hosie et al., 2006; 2007; 2009). The C3α-hydroxyl group is essential for the binding affinity and the receptor-enhancing function by neurosteroids (Harrison et al., 1987; Mitchell et al., 2008). The C20 position ketone (pregnane) or C17 group (androstane) is important for binding affinity of neurosteroids, however receptor modulation is highly reduced for androstane compared to pregnane neurosteroids (Upasani et al., 1997; Reddy and Jian, 2010). These structural regions provide hydrogen bond acceptor and donor interactivity with the transmembrane region of the $GABA_A$ receptor, thereby conferring specificity and potency (Hosie et al., 2007). Hydrogen bonding by the 17β-group does not appear to be critical for channel modulation (Li et al., 2009), however it may be important for the improved potency and efficacy of neurosteroids at synaptic receptors (Gee et al., 1988).

The pharmacophore profile of δ-containing extrasynaptic receptors and the specific structure properties of neurosteroids required to modulate the current-transduction remain unclear. Neurosteroids bind all $GABA_A$ receptors, but they display high affinity for δ-subunit receptors (Brown et al., 2002). Morever, physiological conditions that increase δ-subunit expression enhance neurosteroid sensitivity (Wu et al., 2013). Conversely, conditions of deficient δ-subunit expression reduces sensitivity to neurosteroid modulation (Mihalek et al., 1999; Spigelman et al., 2003; Stell et al., 2003). Allosteric steroid binding to low-efficacy, GABA-gated receptors results in a pronounced conformational change of the channel. This transduction induces greater GABAergic inhibition above partial agonism at δ-containing receptors (Bianchi and Macdonald, 2003). There has not been direct evidence in support of a δ-subunit binding site for neurosteroids. Point mutations to the *Gabrd* gene have been shown to result in reduced $GABA_A$ receptor currents, linked with cases of generalized epilepsies (Dibbens et al., 2004; Feng et al., 2006). Therefore, decreased extrasynaptic inhibitory currents could have cascading effects on seizure susceptibility and pathophysiological restructuring of the brain. Previous structure-activity relationship studies have not investigated the putative tonic current modulation by neurosteroids (Akk et al., 2007; Harrison et al., 1987; Kokate et al., 1994; Qian et al., 2014). Therefore, structure-activity specific for δ-containing extrasynaptic receptors warrants further investigation.

In this study, we sought to determine the structure-activity relationship of neurosteroids at δ-subunit extrasynaptic $GABA_A$ receptors-mediating tonic current in the dentate gyrus, a key structure for the pathophysiology of epilepsy and excitability disorders. We utilized structurally distinct natural and synthetic neurosteroids to assess fractional tonic currents in native, murine DGGCs using hippocampus slice electrophysiology. We also evaluated the structure-activity relationship of neurosteroids for behavioral antiseizure potency in the 6-Hz seizure model in a gender-dependent fashion. It was intended to reveal the key structural features for neurosteroid functional activity at δ-containing $GABA_A$ receptors towards developing a consensus pharmacophore pocket.

II.3 Specific Aim 3

The objective of the third specific aim is to investigate the functional role of tonic inhibiton in the hippocampus epileptogenesis in mice with germline deletion of δ-containing $GABA_A$ receptors.

Currently, there are no specific drugs for preventing or curing epilepsy. The mechanisms underlying the development of acquired epilepsy are not well understood. The term "epileptogenesis" is used to describe the complex plastic changes in the brain that, after a precipitating event, convert a normal brain into a brain debilitated by recurrent seizures (Pitkänen et al., 2009). Limbic epilepsy is caused by diverse cascading factors such as brain injury, stroke, infections, or prolonged seizures. The kindling model has provided a conceptual framework for the idea that "seizures beget seizures" and for developing new molecular targets for preventing epilepsy (Goddard et al., 1969; McNamara et al., 1992). Kindling is the most widely used model of epileptogenesis in which repeated stimulation of limbic structures triggers progressive intensification of behavioral and electrographic seizure activity. The progression of seizures occurs at a subthreshold of motor seizures until propogation advances into later stages of epileptogenic activity. The progressive induction of electrical seizures in the rodent kindling model shares many features with human complex partial seizures (Löscher, 2002). The fully kindled state in animals allows studying the persistence of epilepsy weeks or months after development. In addition, therapeutic interventions can be used to understand disease-modifying criteria necessary for impeding, preventing, or protecting against seizures.

Using a combination of behavioral and pharmacological studies, we explored the progression of kindling epileptogenesis and developmental defects that may alter brain function within the germline δ-subunit knockout mouse model. We hypothesized that mice with a targeted, germline deletion of δ-subunit in the brain exhibit a markedly increased propensity for the development and persistence of kindling epileptogenesis. We provide a rationale for seeking an improved trangenic mouse model under conditional deletion of δ-subunit for further investigation of the functional role of δ-subunit expresssion in the hippocampus.

CHAPTER III
MATERIALS AND METHODS

III.1 Experimental Animals

Animals. Wildtype (WT) adult female C57BL/6 mice, 25 to 30g each were used in this study. $GABA_A$ receptor δ-subunit knockout mice (*Gabrd*$^{-/-}$, δKO) were also used (Mihalek et al., 1999). All strains were maintained on a hybrid C57BL/6-129SV background. All mice were housed four to a cage with access to food and water *ad libitum*. The mice were housed in an environmentally controlled animal facility with a 12 h light/dark cycle. The animals were cared for in strict compliance with the guidelines outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All animal procedures were performed in a protocol approved by the university's Institutional Animal Care and Use Committee.

III.2 Perimenstrual Model of Neurosteroid Withdrawal

A state of perimenstrual-like neurosteroid withdrawal (NSW) hormonal condition was induced in animals by a standard progesterone-finasteride regimen as described previously (Gangisetty and Reddy, 2010), which was based on published protocols for induction of NSW (Smith et al., 1998ab; Moran et al., 1998; Moran and Smith, 1998). Adult female mice were given subcutaneous injection of progesterone (25 mg/kg) twice-daily for seven days. On the final injection, finasteride (50 mg/kg, i.p.) was administered to block 5α-reductase activity for inhibiting progesterone conversion to AP and related neurosteroids. Progesterone was administered rather than allopregnanolone because circulating levels of progesterone, such as those found during the luteal phase, are readily converted to neurosteroids in the brain regions that express neurosteroid synthesizing enzymes (Mellon et al., 2001; Agís-Balboa et al., 2006). The progesterone administration protocol results in a high physiological concentration of allopregnanolone in plasma, and acute withdrawal is evident by a nearly complete decline in allopregnanolone 24 hours after finasteride administration (Gangisetty and Reddy, 2010). Control mice were administered 15% β-cyclodextrin vehicle with the same frequency for the seven day injection period. Experimental studies were carried out 24 hours following the final finasteride or vehicle injection.

III.3 Immunohistochemistry and Confocal Microscopy

Adult mice were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Transcardial perfusion of saline followed by 4% paraformaldehyde was completed to preserve the rodent brain.

Immunohistochemistry. Following cryoprotection of brain, the tissue was rapidly frozen in isopentane pre-cooled to -70˚C. The frozen tissue was then cut in 20 µm tranverse sections on a cryostat and mounted on gelatin-coated slides. Sections were processed for distribution of $GABA_A$ receptor δ-subunit using a specific *Gabrd* primary antibody (1:250) (Millipore, Billerica, MA), according to the avidin-biotin-complex method (Hsu et al., 1981). Technical assitance was provided by FD Neurotechnologies (Maryland).

Immunocytochemistry and confocal microscopy. The δ-subunit distribution in the hippocampal neurons was determined by immunocytochemistry (Mangan et al., 2005; Wu et al., 2013). Acutely dissociated hippocampal CA1 pyramidal cells (CA1PCs) and dentate gyrus granule cells (DGGCs) from adult, female mice in the vehicle and NSW groups were fixed with 4% paraformaldehyde for 15 min followed by several glycine-PBS washes. Cells were permeabilized with ice-cold methanol for 3 min at 4 °C followed again by several rinses with PBS. Cells were then incubated with blocking solution containing 1% bovine serum albumin (Vector Laboratories, UK), 2.5% normal goat serum and 0.1% Triton X-100 for 1 h. After the blocking, the cells were incubated together with primary rabbit $GABA_AR$ δ-subunits (1:100, PhosphoSolutions) or control rabbit IgG (1:100, Santa Cruz Biotechnologies) for 1 h at room temperature. Samples were rinsed and then incubated with Alexa Fluor® 555 labeled secondary antibodies of goat-anti-rabbit IgG (1:200, Molecular Probes, Invitrogen) for 1 h in the dark, washed extensively, and treated with ProLong AntiFade (Molecular Probes, Invitrogen). Serial image sections through focus with step size of 0.1-0.3 µm thickness were collected and analyzed using Nikon confocal microscope with NIS-Elements software suite (Nikon Instruments Inc., Melville, NY). The parameters for the confocal microscopy included pinhole =1 AU, the pixel dwell time =0.5 frame/sec, gain (HV) at same level for sample and control IgG, power for the laser = 5%, objective = Plan Apo VC 60x Oil DIC N2 (Magnifying factor = -1.00, numerical

aperture (NA)=1.40, refractive index (RI)=1.51, Olympus, Tokyo, Japan).The normalized mean intensity was used as the ratio from mean intensity of $GABA_A$ receptor δ-subunits minus background *to* mean density of control antibody minus background. Mean intensity is a ratio of integrated density of the signal divided by region of interest. Then normalized mean intensity from treatment group (NSW group) was used to compare with normalized mean intensity from control group.

Western blot analysis. Western blot analysis of $GABA_A$ receptor subunit protein expression in the hippocampus was performed using affinity-purified rabbit polyclonal antibodies for $GABA_A$ receptor δ-subunit (PhosphoSolutions, Aurora, CO). Hippocampi were homogenized in RIPA buffer (Pierce), and the extracted protein (100 μg) was loaded onto 10% Tris-HCl gels and subjected to electrophoresis. Blots were then transferred to a polyvinylidene fluoride membrane (Bio-Rad). Membranes were blocked in 5% nonfat milk at room temperature for 1 h. Membranes were then incubated with $GABA_A$ subunit-specific antibody (1:1000) or a mouse monoclonal β-actin antibody (1:1000) at 4°C overnight. Membranes were washed and incubated with an anti-rabbit antibody (1:1000) conjugated to horse radish peroxidase for 1 h at room temperature. Receptor subunit bands were detected using enhanced chemiluminescence reagent (Perkin-Elmer, Shelton, CT). Protein bands were quantified using Alpha Imager software (Alpha Innotech, San Leandro, CA). All values were normalized to β-actin expression in the same samples to control for loading amount variability and then expressed as a percent change with respect to mean control values. Brain tissue samples were collected from a group of 6-10 mice for each treatment.

Timm staining procedure. Timm staining was conducted as described previously (Cavazos et al., 1991). Mice were deeply anesthetized with ketamine/xylazine mix and transcardially perfused with 75 mL 0.9% saline solution followed by 100 mL 1% sodium solfide solution. Perfusion of 100 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (7.4 pH) followed, and a final perfusion with 50 mL 1% sodium sulfide was carried out. The brain was dissected and postfixed in 4% paraformaldehyde overnight at 4˚C. Brains were then processed with phosphate buffer and sucrose treatment and cut in 20 μm transverse sections with a cryostat. Slices were then dry mounted onto slides and allowed to dry overnight. Gum arabic, citrate buffer, hydroquinone, and silver lactate reagents were mixed mechanically on the day of staining. Mounted slides were

uniformly soaked in the Timm stain in dark for 3.5 hours. Slides were then washed with distilled water and counter-stained with 0.1% cresyl violet, if desired. Slides were permanently fixed with DPX mountant (Sigma) and allowed to dry before imaging and analysis.

Densitometric analysis. Staining intensity for Timm and δ-subunit antibody histology was quantitatively measured by densitometric analysis. Densitometry was completed in hippocampus regions of interest with Photoshop software. Mean density of gray-scale staining was normalized to area and white background. Densitry scores were then non-parametrically graded using a linear scale.

III.4 Electrophysiology Studies

Hippocampal slice preparation. Transverse slices (300 μm thickness) of hippocampus were prepared using standard techniques from adult mice. Mice were anesthetized with isoflurane, and brains were excised rapidly and placed in cold (4 °C) artificial cerebrospinal fluid (ACSF) buffer containing 0.3 mM kynurenic acid (Tocris Bioscience, MN). ACSF buffer was composed of (in mM): 126 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 11 glucose (pH adjusted to 7.35-7.40 with 95% O_2 – 5% CO_2, 305–315 mOsm/kg). Hippocampal slices were cut with a Vibratome in cold ACSF (model 1500 with 900 Refrigeration System; Leica Microsystems, Inc., Bannockburn, IL). For electrophysiology and immunocytochemistry studies, microdissected subfield hippocampal slices were equilibrated in ACSF at 24°C and continuously bubbled with oxygen (95% O_2-5% CO_2). For each electrophysiology experiment, 3-4 animals were used for each group and drug concentration tested.

Dissociation of neurons. Hippocampus CA1PC and DGGC dissociation was prepared by the standard dissociation technique described previously (Kay and Wong, 1986; Reddy and Jian, 2010; Wu et al., 2013). The hippocampal pieces of the CA1 or DG region were microdissected carefully under a microscope (model SMZ 647; Nikon, Tokyo, Japan) and incubated in ACSF for 1 h at 24°C. The isolated slices were transferred into an enzymatic solution consisting of ACSF with protease XXIII (3 mg/ml, Sigma-Aldrich, St. Louis, MO). The slices were then incubated for precisely 23 to 25 min at 24°C. The remaining slices were rinsed twice with ACSF and gently triturated through three increasingly smaller, fire-polished Pasteur pipettes to yield

single cells. For each batch, slices were triturated five or six times with each pipette in approximately 1 ml of ACSF. Then, the solution was allowed 1 min for the tissue to settle down, and the suspension of freshly dispersed cells were carefully plated onto the recording chamber (Warner Instruments, Hamden, CT) for electrophysiology and immunocytochemistry experiments.

Recording of GABA-evoked currents. Electrophysiological recordings were performed in the whole-cell patch-clamp configuration (Reddy and Jian, 2010; Wu et al, 2013). All electrophysiological experiments were performed at 22-24 °C. The recording chamber was fixed into the stage of an inverted microscope with phase-contrast and differential interference contrast optics (model IX71; Olympus, Tokyo, Japan). The physiological bath solution for whole-cell recording had the composition (in mM): 140 NaCl, 3 KCl, 10 HEPES, 2 $MgCl_2$, 2 $CaCl_2$, and 16 glucose (pH adjusted to 7.4 with NaOH, osmolarity, 315–325 mOsm/kg). Cells were visualized and images were acquired through video camera CCD-100 (Dage-MTI, Michigan City, IN) with FlashBus Spectrim 1.2 software (Pelco, Clovis, CA). Recording pipettes were pulled from capillary glass tubes (King Precision Glass, Claremont, CA) using a P-97 Flaming-Brown horizontal puller (Sutter Instrument Company, Novato, CA). The pipette tip resistances were 2 to 4 MΩ for single cell recording and 4 to 6 MΩ for slices recording. The recording pipettes were filled with a cesium pipette solution containing (in mM): 124 CsCl, 20 tetraethylammonium, 2 $MgCl_2$, 10 EGTA, 10 HEPES, 0.1 GTP, 4 ATP (pH adjust to 7.2 with CsOH, osmolarity, 295–305 mOsm/kg). In slice recordings, 5 mM lidocaine *N*-ethyl bromide (QX-314) was added to the pipette solution. Currents were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). The membrane capacitance, series resistance, and input resistance of the recordings were monitored by applying a 5-mV (100-ms) depolarizing voltage step from a holding potential of -70 mV for dissociated cells and -65 mV for slice recordings. Signals were low-pass filtered at 2 kHz and digitized at 10 kHz with Digidata 1440A system. The current values were normalized to cell capacitance (an index of cell size) and expressed as current density (pA/pF). For whole cell current from isolated single cells, fractional potentiation produced by AP was calculated as I_A/I_{GABA}, where I_{GABA} was the response of peak amplitude at the application of GABA (3 μM) and I_A is the response of peak amplitude at the co-application of GABA and the neurosteroid AP (0.01 to 1 μM). For fast application of test drugs, the perfusion pipette was positioned <200 μm away from the cell in the dish. GABA,

allopregnanolone, and $GABA_AR$ competitive antagonist bicuculline (10 μM) were applied using a multi-channel perfusion system (Automate Scientific, Berkeley, CA).

Tonic current recording and analysis. The $GABA_A$ receptor mediated tonic current recording and analysis were made as described previously (Mtchedlishvili and Kapur, 2006; Wu et al., 2013). Hippocampal slices (300 μm) for were maintained in continuously oxygenated ASCF at 32°C in a holding chamber for 60 min, and then recorded from were made at room temperature. Hippocampal CA1PCs and DGGCs were visually identified with an Olympus BX51 microscope equipped with a 40x water-immersion objective, infrared-differential interference contrast optics, and video camera (Kay and Wong, 1986). Tonic current and phasic, miniature inhibitory postsynaptic currents (mIPSC) of $GABA_ARs$ were recorded in the presence of tetrodotoxin (TTX, 0.5 μM, Na^+ channel blocker and inhibition of action potential-evoked neurotransmitter release), D,L-2-amino-5-phosphonovaleric acid (APV, 40 μM, N-methyl-D-aspartate channel blocker, Sigma), and 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 μM, non-N-methyl-D-aspartate glutamate receptor blocker). The competitive antagonist gabazine (SR-95531, 50 μM) was added to perfusion after slice recordings to confirm block of GABAergic currents.

Off-line current analysis was performed with pClamp 10.2 software (Molecular Devices, Sunnyvale CA) and in-house software. To study the tonic inhibition, transient events were manually removed from the current trace, so that it consisted only of membrane current in the voltage-clamp mode (Mtchedlishvili and Kapur, 2006; Wu et al., 2013). Averaged amplitude of tonic current shift in conductance and root-mean-square noise amplitude were measured. The $GABA_A$ receptor tonic current was expressed as the outward shift in holding current after application of gabazine (50 μM). Currents for a single cell were normalized to membrane capacitance for that cell (pA/pF). The amplitude of root-mean-square (I_{rms}) is the noise conductance from chloride ions passing through the opened channels and in proportion to the chloride driving force. I_{tonic} was measured and averaged in 100 ms each epoch with 1 sec interval between epochs for 30 epochs. The measurements were taken 30 s before and 2-3 min after application of a drug. I_{rms} was studied in 50 ms each epoch with 500 ms interval between epochs for 30 epochs before and after drug application in each cell. To assess the effect of a drug on I_{rms} in an individual neuron, the distribution of I_{rms} in 30 epochs before the application of a drug (during the baseline period) was compared with that after drug application by a Student's

independent t-test. To compare data obtained from a group of neurons, I_{rms} values in individual epochs before and after drug application were averaged. Changes in I_{tonic} or I_{rms} are expressed in pA of current.

For measurement of desensitization of extrasynaptic current, traces were fit using a standard exponential function ($f(t) = A * e^{-t/\tau} + C$) with the Chebyshev fitting method in Clampfit 10.2 software (Molecular Devices, Sunnyvale, CA) with $r^2 \geq 0.95$. The asymptote of desensitization was described with regression analysis. The time point reaching within 10% of the arbitrary finite limit was designated the beginning time point measurement for the desensitized tonic current, averaged in 100 ms epochs with 1 sec intervals between epochs. Percent change of tonic current at the peak and desensitized points was then compared.

Miniature postsynaptic current data analysis. mIPSCs were detected using off-line analysis with Mini-Analysis software (Synaptosoft, Leonia, NJ), with the threshold for detection set at least three times the baseline root mean square noise. mIPSC characteristics of amplitude, frequency, and decay time constants were determined and compared between groups as described previously (Dubois et al., 2013), adapted for granule cells. mIPSCs were acquired for 3 min for each drug response and condition. The decay of averaged mIPSC within dentate gyrus granule cells was best fit with a double exponent time constant (Stell et al., 2003). Non-overlapping events with single-peaks were used to generate an ensemble average mIPSC by aligning the rising phase, and the 10-90% decay phase for each neuron and fit with the bi-exponential function: $I(t) = A_1 * e^{(-t/\tau 1)} + A_2 * e^{(-t/\tau 2)}$, where A_1 and A_2 are the fast and slow component amplitudes, and τ_1 and τ_2 are their respective time constants. A mean weighted decay constant was determined as $\tau_w = (A_1 * \tau_1 + A_2 * \tau_2) / (A_1 + A_2)$, as previously specified (Sun et al., 2007). The mean values in response to drug application were established for each recorded neuron, and the results are expressed as mean ± S.E.M. for each group, with comparisons between groups made using Student's t-test. Comparisons of cumulative probability distributions were made using the Kolmogorov-Smirnov test. Results were considered significant if the two-tailed p values were < 0.05.

III.5 Behavioral Studies

Hippocampus kindling seizures. The rapid kindling model of epileptogenesis was utilized for assessment of seizure susceptibility in case of the NSW model. This model allows accelerated evaluation of experimental manipulations during the progression of seizure induction (Reddy and Mohan, 2011; Wu et al., 2013). For checking the rate of epileptogenesis in knockout mice, a standard once-daily stimulation protocol was adapted.

Electrode implantation and stimulation procedures for mouse hippocampus kindling were performed as described previously (Reddy and Mohan, 2011). Mice were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg. i.p.) and xylazine (10 mg/kg, i.p.). A stimulation-recording bipolar electrode (model MS303/1; Plastics One, Roanoke, VA) was stereotaxically implanted in the right ventral hippocampus (2.9 mm posterior, 3.0 mm lateral, and 3.0 mm below dura) using the Franklin and Paxinos atlas (1997). The electrode was anchored with dental acrylic to three small screws placed in the skull. After a postoperative recovery period of at least 1 week, the electrographic afterdischarge threshold was determined by an application of 1 ms duration of biphasic rectangular pulses at 60 Hz for 1 s, beginning at 25 μA by using an isolated pulse stimulator (A-M Systems, Sequim, WA). Afterdischarge duration was the total duration of hippocampus electrographic spike activity (amplitude > 2 x baseline) occurring in a rhythmic pattern at a frequency >1 Hz. Additional stimulations increasing in increments of 25 μA were given at 5 min intervals until an electrographic afterdischarge duration lasting at least 5 s was detected using the digital EEG system (Astro-Med, West Warwick, RI). For subsequent stimulations, mice were stimulated at 125% afterdischarge threshold (1 ms duration pulse, 60 Hz frequency for 1 s). Two different stimulation protocols were used, 1) rapid kindling for seizure progression in neurosteroid withdrawal paradigm, or 2) regular, daily kindling for standard epileptogenesis. For rapid kindling experiements, mice were stimulated at 30-min intervals until they showed stage 5 motor seizures. Stimulations were delivered every 30 min until stage 5 seizures were elicited on 3 consecutive trials. For allopregnanolone treatment studies and regular kindling epileptogenesis tests, mice were stimulated once per day until stage 5 seizures were elicited on 3 consecutive days. In either case of kindling epileptogenesis, stimulations were iterated until each mouse reached stage 5 seizures, which is considered the fully-kindled state (Reddy and Mohan, 2011). The electrographic activity and afterdischarge

duration were acquired from the hippocampal electrode using Axoscope 8.0 software with Digidata 1322A interface (Molecular Devices, Sunnyvale, CA) through a Grass CP511 preamplifier (Astro-Med, West Warwick, RI). Behavioral seizures were rated according to Racine's scale (Racine, 1972) as modified for the mouse: stage 0, no response or behavior arrest; stage 1, chewing or facial twitches; stage 2, chewing and head nodding; stage 3, forelimb clonus; stage 4, bilateral forelimb clonus and rearing; stage 5, rearing and falling. During each stimulation session, the behavioral seizure score and the afterdischarge duration were recorded. Rate of kindling development, that is numbers of stimulation required to induce stage 5 seizures, was determined in rapidly kindled mice subjected to NSW. Cumulative afterdischarge duration was calculated as an index of total seizure activity required to reach stage 5 seizure. Kindling experiments were conducted in a group of mice consisting of 6-15 mice for each treatment or genotype.

Six-Hertz seizure model. The 6-Hz model of partial seizures was used according to previously described protocol (Barton et al., 2001, Kaminski et al., 2004). WT mice were stimulated with a constant-current device delivered via corneal stimulation with 0.2 ms-duration monopolar rectangular pulses at 6 Hz for 3 seconds (World Precision Instruments, Sarasota, FL). Ocular anesthetic (0.5% tetracaine) was administered as an anti-pruritic to the corneas 10 min before stimulation. Immediately prior to stimulation, the corneal electrodes were wetted with 0.9% saline solution. Mice were manually restrained during the 6-Hz stimulation, and they were then immediately released into an observation chamber. Seizures were frequently preceded by a brief period of locomotor convulsion and hunched posture. Animals exhibited various combinations of stunned posture, rearing (bipedal standing), forelimb automatic movements, clonus, twitching of vibrissae, and Straub-tail. The seizure activity duration ranged from 20 to 40 sec in untreated, control animals. The resumption of normal exploratory behavior signified the completion of the seizure. For CC_{50} determination (current producing seizures in 50% of animals), different current intensities in the range of 6 to 44 mA were administered to control groups of animals. 32 mA was determined to elicit seizures in 100% population of male and female C57BL/6-129SV (WT) control mice. A fixed current of 32 mA was delivered as the standarized intensity for comparing all mice across structure-activity responses of drugs. In treatment studies, protection from seizure activity was designated as the experimental end point of anticonvulsant treatment as maximally effective dose. Animals were considered to be protected if they resumed exploratory behavior

within 10 sec of the stimulation. Eight to ten animals were used for each dose of anticonvusant drug treatment.

Pentylenetetrazol seizure model. Mice were injected with pentylenetetrazol (PTZ) subcutaneous injection and monitored for seizures as adapted from a previous PTZ seizure susceptibility study (Ferraro et al., 1999). Latencies to focal (partial clonic), generalized (generalized clonic), and maximal (tonic-clonic) behavioral seizures were recorded. Three mice were observed simultaneously with seizure latencies derived using individual digital timing devices. A dose-response was compiled with male and female mice from both WT and δKO strains in order to determine a dose of PTZ that best distinguished their seizure sensitivity. At the end of the 30 min. observation period, mice were killed by cervical dislocation. Four stages of seizure behavioral response to subcutaneous PTZ injection were defined as follows: 1) hypoactivity characterized by progressive decrease in motor activity until the animal came to rest in a crouched or prone position, 2) myoclonic and jerking spasms characterized by brief focal seizures lasting 1 sec or less, 3) generalized clonus characterized by sudden loss of upright posture, all four limb and tail clonus, rearing, and autonomic signs, and 4) tonic-clonic seizure characterized by generalized seizure followed by tonic hindlimb extension. Six to eight mice were used per group. Latencies to each seizure stage were summed to assign each mouse a seizure score that was used as a quantitative trait measure for dose-response according to the equation:

Seizure score = (0.2) * (1/partial clonus latency) + (0.3) * (1/generalized clonus latency) + (0.5) * (1/tonic-clonic latency)

The weighting factors (0.2, 0.3, and 0.5) were included as a means of incorporating a measure of the progressive nature of the PTZ-induced seizure phenotype into the severity rating because generalized clonus is regarded as a more significant event than partial clonus, and tonic hindlimb extension is regarded as the most severe component of the phenotype. In this way, the seizure score reflects the degree of progression of the seizure syndrome in each mouse, as previously described (Ferraro et al., 1999).

Porsolt forced swim (depression) test. The latency to first immobility and the total duration of immobility of mice were measured using the Porsolt's forced swim test (Porsolt et al., 1978). Each mouse was placed individually in a glass cylinder (21 x 12 cm) containing 9 cm of water at

22 ± 1 °C. The immobility time was recorded during a 6 min forced swimming test. Each mouse was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water. To assess any differences in strain, adult WT and δKO mice were compared, as well as male and female of each strain.

Elevated plus maze test. Cognitive behavior was evaluated by using the elevated plus-maze learning task (Itoh et al., 1990), which measures spatial long-term memory. Transfer latency (the time in which the mouse takes to move from the open arm to the enclosed arm) was utilized as an index of learning and memory processes. The apparatus consisted of two open arms (30.5 x 7.6 cm) and two enclosed arms (30.5 x 7.6 x 22.8 cm) elevated to a height of 30 cm from the floor. The test consisted of placing a mouse end an open arm facing away from the center and the transfer latency was recorded on the first day. If the mice did not enter the closed arm within 90 s, they were placed into the closed arm for exploration. In this case, the transfer latency was recorded as 90 s. The animals were allowed to explore the apparatus for 60 s to become acquainted with the maze. On the second day, 24 h after the first exposure, transfer latency was again recorded. Each animal was used only once for acquisition and retention trials. Allopregnanolone (1 mg/kg, s.c.) was administered 15 min prior to the assessment of the first day trial in the treatment group. An increase in acquisition/learning processes was defined as a decreased transfer latency on the second day trial relative to the first day trial. Failure to decrease the transfer latency on the second day trial was interpreted as an impairment of learning process. The test platform was wiped down with a damp cloth after each trial. Mice which fell off the maze were exluded from the experiment.

III.6 Drugs and Reagents

Electrophysiology reagents. All chemicals used in the electrophysiology studies were purchased from Sigma-Aldrich unless otherwise specified. Stock solutions were diluted in the external perfusion solution to the desired concentration for electrophysiological use. Allopregnanolone (3α-hydroxy-5α-pregnan-20-one, AP), THDOC (3α,21-dihydroxy-5α-pregnan-20-one), ganaxolone (3α-hydroxy-3β-methyl-5α-pregnan-20-one, GNX), 5β-AP (3α-hydroxy-5β-pregnan-20-one), 3β-AP (3β-hydroxy-5α-pregnan-20-one), androstanediol (3α-hydroxy-5α-

androstane-17β-diol, AD), androsterone (3α-hydroxy-5α-androstane-17-one, AN), etiocholanolone (3α-hydroxy-5β-androstane-17-one, ETIO), alfaxolone (3α-hydroxy-5α-pregnan-11,20-dione, ALFX), and ORG-20599 (2β,3α,5α-21-Chloro-3-hydroxy-2-(4-morpholinyl)pregnan-20-one), and DS2 (4-chloro-*N*-[2-(2-thienyl)imidazo[1,2-a]pyridin-3-yl]benzamide) were prepared in dimethyl sulfoxide for electrophysiology experiments. The concentration of dimethyl sulfoxide in final solution was less than 1%. Neurosteroids, progesterone, and finasteride (*N*-(1,1-dimethylethyl)-3-ox-(5α,17β)-4-azaandrost-1-ene-17-carboxamide) were acquired from Steraloids (Newport, RI). Stock concentrations of GABA, SR-95531, bicuculline methiodide, APV (D,L-2-amino-5-phosphonovaleric acid), DNQX (6,7-dinitroquinoxaline-2,3-dione), TTX (tetrodotoxin), and THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol hydrochloride) were dissolved in water. Kynurenic acid, DS2, GNX, and THIP were acquired from Tocris. TTX was acquired from Calbiochem.

Drug injections. Progesterone (25 mg/kg) and finasteride (50 mg/kg) solutions were made in 15% β-cyclodextrin w/v in water for the neurosteroid withdrawal paradigm. For behavioral experiments involving neurosteroids, drug was dissolved in 0.9% NaCl and suspended in 20% β-cyclodextrin. Drugs were administered to animals subcutaneously or intraperitoneally in a volume equaling 1% of the animal's body weight.

III.7 Statistical Analysis

Group data were expressed as the mean ± S.E.M. For whole-cell current recordings, fractional potentiation produced by neurosteroid was calculated as I_{NS}/I_{GABA}, where I_{GABA} is the peak current amplitude of the control GABA response and I_{NS} is the peak current response of the co-application of GABA and the allosteric drug. A control GABA concentration of 3 μM evoked 10% of the maximal inhibitory current (EC_{10}), as determined previously in native, murine CA1 pyramidal cells (Reddy and Jian, 2010) and DGGCs (Wu et al., 2013). The concentration of allosteric modulator producing half of the maximal increase in the amplitude of the GABA response (EC_{50}) was determined by fitting the concentration-response relationships to the following nonlinear sigmoid Hill function: $\mathbf{I/I_{max} = [1 + (EC_{50} / [A])^n]^{-1}}$, where **A** is the allosteric modulator concentration, $\mathbf{I_{max}}$ is the current evoked by GABA in the presence of a maximal potentiating concentration of allosteric modulator, **I** is the current produced by GABA in the

presence of a concentration **A**, $\mathbf{EC_{50}}$ is the concentration of A required to produce half of its own maximal GABA potentiating effect, and ***n*** is the Hill coefficient.

For electrophysiology and pharmacology studies, concentration-response curve data were subjected to non-linear, logistic fitting. A Hill curve fitting was acquired only for concentration-responses that achieved a plateau in maximum current response. Statistical comparisons of parametric measures including electrophysiology and expressional data were performed using an independent two-tailed Student's t-test followed by Tukey's HSD test *post hoc*. In all statistical tests, the criterion for statistical significance was $p < 0.05$, unless otherwise specified.

In case of behavioral observations including kindling data, group data were expressed as mean ± S.E.M. The Kruskal-Wallis ANOVA and Mann-Whitney U test were used to analyze significant differences in seizure stages between gender and genotypes. Significant differences in afterdischarge durations between genotype and treatment groups were assessed by the Student's upaired t-test, repeated measures ANOVA, or one-way ANOVA followed by Dunnett's test. Dose-response curves and their ED_{50} values in the 6-Hz test were analyzed for significance using the Litchfield and Wilcoxon test. Differences were considered statistically significant at $p < 0.05$.

CHAPTER IV

RESULTS*

IV.1 Plasticity and Function of δ-containing $GABA_A$ receptors in the Hippocampus in a Perimenstrual Model of Neurosteroid Withdrawal

IV.1.1 NSW induces up-regulation of $GABA_A$ receptor δ-subunit expression

To simulate the perimenstrual-like hormonal milieu in mice, we first created a condition of prolonged, elevated progesterone and neurosteroids to model the luteal phase and then induced an abrupt decline in neurosteroid levels to more closely model perimenstrual changes in women (**Figure 6**). This paradigm is consistent with perimenstrual catamenial epilepsy in which patients experience neurosteroid withdrawal (NSW) (Reddy et al., 2012).

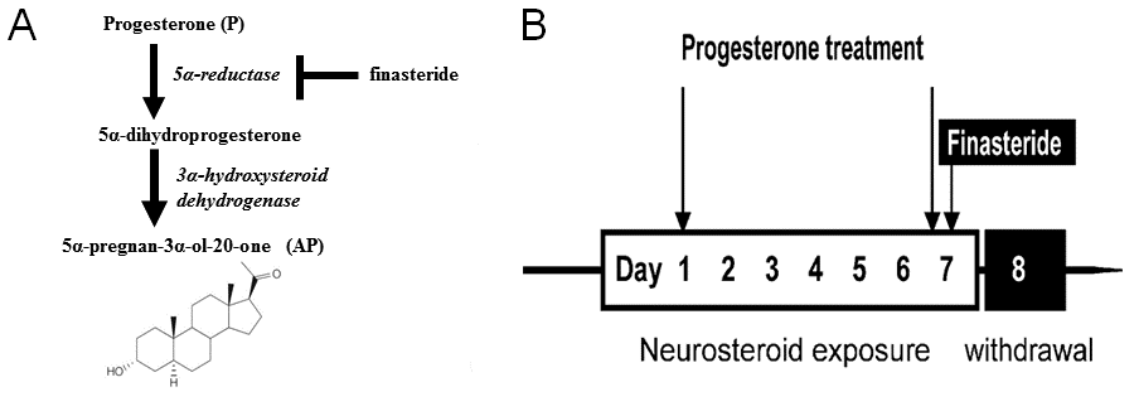


Figure 6. Overview of AP synthesis and perimenstrual-like NSW paradigm. ***A***, Abrupt inhibition of 5α-pregnan-3α-ol-20-one (allopregnanolone; AP) synthesis was accomplished by pharmacological blockade of 5α-reductase activity with finasteride in adult, female mice. ***B***, In the perimenstrual model, progesterone was administered twice-daily for seven days and finasteride was administered on the final day, resulting in systemic and brain neurosteroid withdrawal. Molecular, electrophysiology, and behavioral studies were conducted 24 hours following induction of withdrawal.

*Reprinted with permission from "Perimenstrual-like hormonal regulation of extrasynaptic δ-containing $GABA_A$ receptors mediating tonic inhibition and neurosteroid sensitivity by Carver CM, Wu X, Gangisetty O, Reddy DS, 2014. *Journal of Neuroscience*, 34(43), 14181-14197, Copyright [2014] by Carver, Wu, Gangisetty, and Reddy.

To determine the withdrawal-induced changes in δ-subunit plasticity in the hippocampus, δ subunit expression was explored. Protein levels of δ-subunit in the DG were increased in NSW animals relative to controls as detected by western blot analysis with a δ-specific antibody (relative protein expression: 1.00 ± 0.11 control vs. 4.56 ± 0.22 NSW, $p = 0.0001$, **Figure 7**).

Immunohistochemistry was carried out on brain slices from NSW and control mice. Brain tissue slices were processed with the *Gabrd* antibody, specific for the $GABA_A$ receptor δ-subunit in a 1:250 fraction. Brain sections derived δKO mice did not produce exhibit staining with the *Gabrd* antibody. Images of the hippocampus were collected and analyzed with densitometry using Photoshop software (**Figure 8A**). Staining densities from the CA1 pyramidal layer, stratum radiatum, and DG molecular and granule layer subregions were quantified (**Figure 8B**). NSW WT mice displayed significantly greater antibody staining intensity than control WT mice in the DG molecular layer. No antibody staining was detected from NSW or control δKO mice. Overall, these findings indicate a marked increase in the extrasynaptic expression of δ-subunit in the dentate gyrus after NSW in the mouse perimenstrual-like paradigm.

To confirm the elevation of δ-containing receptors within the hippocampus, we visualized and determined the single cell distribution of δ-subunit by fluorescent immunocytochemistry using a δ-specific primary antibody. Staining with the antibody showed broad distribution of δ-subunit on the soma, axon, and dendritic regions of acutely dissociated CA1 pyramidal cells and DGGCs acquired from NSW and control mice (**Figure 8C**). The normalized mean intensity, expressed as percent change in fluorescence mean intensity of δ-subunit staining relative to the mean intensity of control IgG (see Method section III.3), was significantly greater in DGGCs of NSW mice as compared to control animals (**Figure 8D**, $p < 0.05$). Percent change of immunofluorescence between control and NSW DGGC (77.1 ± 1.3 %) was significantly greater than the change in CA1 cells due to NSW (13.4 ± 2.1 %; $p < 0.05$). The δ-subunit protein was concentrated on cell membrane from X-Z and Y-Z axes obtained from confocal microscopy, as demonstrated previously (Wu et al., 2013).

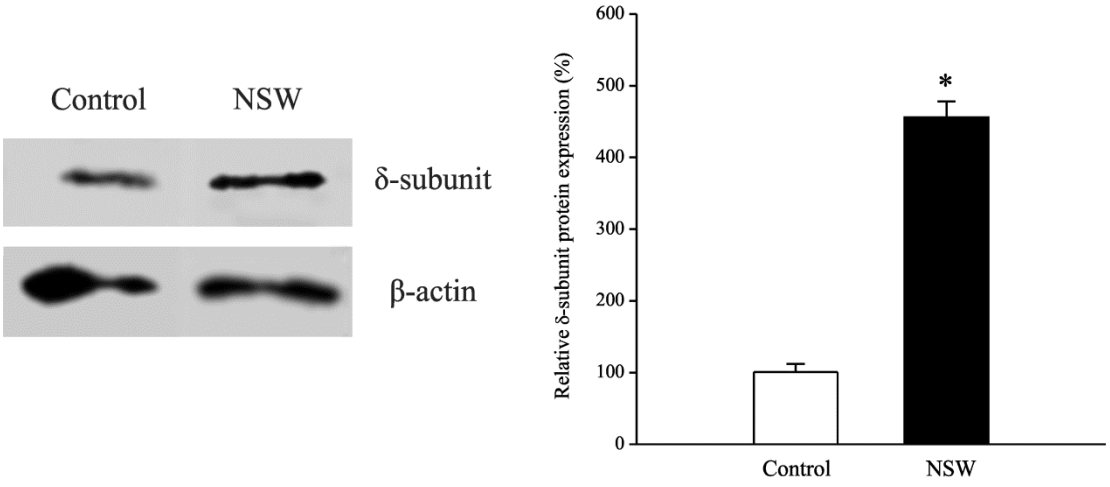
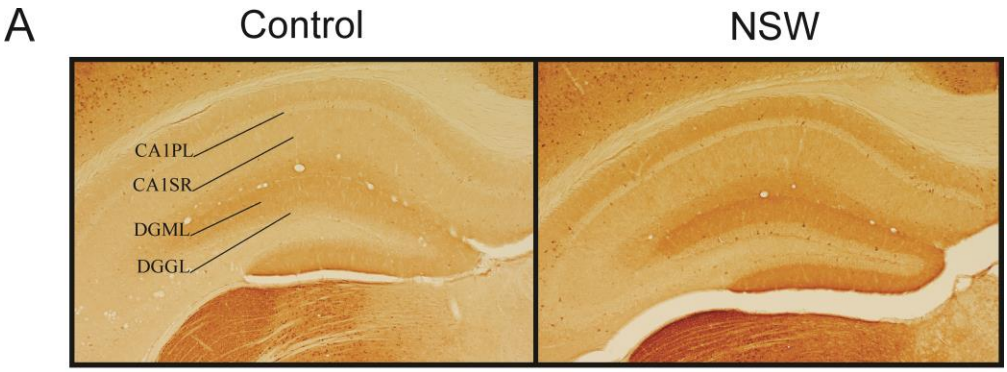


Figure 7. Up-regulation of $GABA_A$ receptor δ-subunit expression in the hippocampus of NSW mice. Control and NSW protein levels as detected by western blot of protein using a δ-subunit-specific 1˚ antibody. Protein loaded was normalized to amount of β-actin. NSW increased δ-subunit protein levels in DG compared to control animals as denoted by representative western blot analysis and relative quantification. * $p < 0.05$ vs. control (n = 6-10 mice per group).



B

Hippocampus Subregion	Control	NSW	Mean Percent Change
CA1PL	+	++	7.6%
CA1SR	+	++	8.1%
DGML	++	++++	20.4%
DGGL	+	++	7.5%

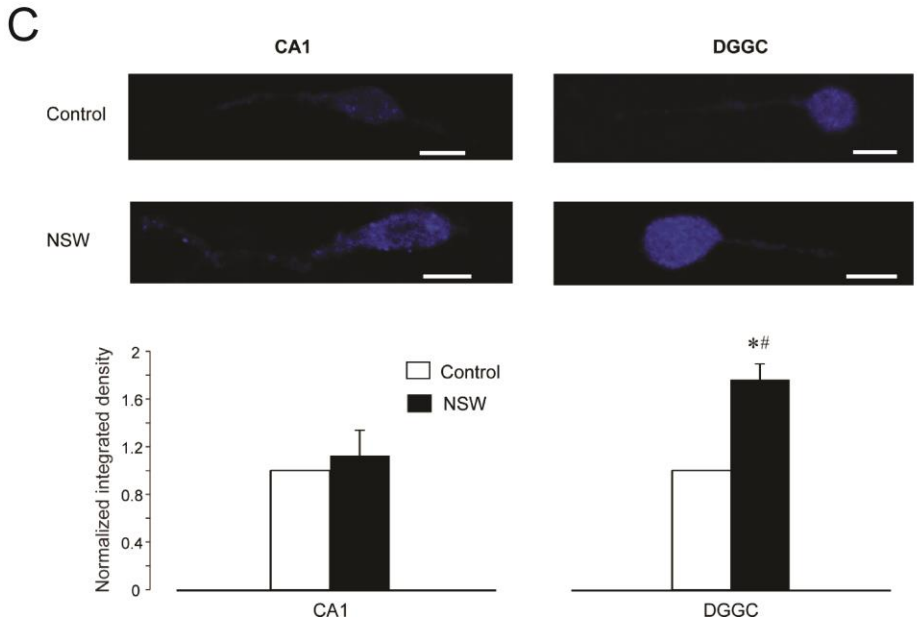


Figure 8. Distribution of δ-subunit in hippocampal CA1 neurons and DGGCs in control and NSW mice. ***A***, Mouse hippocampus tissue staining for δ-subunit-specific antibody (1:250) from control and NSW mice. ***B***, Quantification of densitometric distribution of δ in the hippocampus subregion ROI stratum radiatum (CA1SR), pyramidal layer (CA1PL), dentate granule cell layer (DGGL), and the dentate molecular cell layer (DGML). The DGML displayed robust increase to density of δ-subunit in the dendritic region of granule cells and had significantly greater percent change to antibody staining (n = 8 – 10 slides per group). ***C***, Immunocytochemistry distribution of CA1 vs DGGC principal cells. DGGCs displayed significantly greater normalized integrated density of antibody fluorescence in NSW compared with controls. Bar = 10 μm. * $p < 0.05$ vs. control; # $p < 0.05$ vs. CA1 in NSW (n = 6-24 cells per group).

The lack of significant increase to δ-subunit expression in CA1 is contradictory to previous findings of increased δ and α_4 in CA1 of steroid withdrawn pubertal mice (Shen et al., 2007). However, our findings represent plasticity in adult females, and the discrepancies could be related to age differences between the mouse models used or other experimental factors. From mouse neonates to adulthood, granule cells undergo increased GABA and AP affinity and potency, denoting substantial $GABA_A$ receptor expressional changes across development (Mtchedlishvili et al., 2003). Our data is consistent with the expression pattern of δ-subunit, which is expressed in relative abundance in dentate gyrus compared to other hippocampus subfields (Brickley and Mody, 2012).

IV.1.2 NSW confers enhanced sensitivity to AP potentiation of whole-cell GABA-gated currents in DGGCs

To confirm a functional role for NSW-induced expression of δ-containing $GABA_A$ receptors, we recorded whole-cell, GABA-gated currents in acutely dissociated DGGCs and CA1 pyramidal neurons with voltage-clamp patch electrophysiology (**Figure 9**). There are previous reports of δ-subunit-containing receptors with sub-micromolar GABA affinities (Mortensen et al., 2010; Meera et al., 2011); however, these aforementioned studies were conducted in heterologous expression systems using recombinant $GABA_A$ receptors. The effective concentration value depicted in the dissociated experiments in Figure 9 was derived from concentration-response curves for native, murine hippocampal neurons (Reddy and Jian, 2010; Wu et al., 2013). This range of GABA affinity is consistent with previous reports within adult mouse granule cells in which the GABA EC_{50} is between 18-20 μM and the EC_{10} value is greater than 1 μM (Mtchedlishvili et al., 2003). 3 μM GABA is at the upper limit of the detected range of extracellular GABA *in vivo* (Lerma et al., 1986), and δ-containing receptors shift from low-efficacy to high-efficacy gating upon neurosteroid interaction (Bianchi and Macdonald, 2003). Thus, 3 μM GABA (EC_{10}) was applied to establish a control agonist current for each cell without producing significant receptor desensitization (Reddy and Jian, 2010). Increasing concentrations of AP (0.01 to 1 μM) were then co-applied with 3 μM GABA. A two minute washing interval was implemented between each neurosteroid application for adequate drug removal and minimization of receptor desensitization.

AP elicited a concentration-dependent potentiation of GABA current in both CA1 and DGGC (**Figure 9A**). These currents were blocked by bicuculline and gabazine, indicating that they are mediated by $GABA_A$ receptors. Rapid application of AP in the absence of GABA did not produce gating of current. Allosteric potentiation of receptors by AP was measured as fractional potentiation I_A/I_{GABA}, where I_{GABA} was the peak current amplitude response to 3 μM GABA alone, and I_A was the peak current amplitude of GABA co-applied with AP (Wu et al., 2013). Data were compiled from 8 to 16 cells for each AP concentration and neuron type. CA1 pyramidal cells from control and NSW animals displayed similar levels of AP-mediated fractional potentiation with no significant differences between conditions at any of the tested concentrations (**Figure 9B**). In NSW DGGC, AP (0.1 to 1.0 μM) produced a greater potentiation of GABAergic current than in the vehicle control DGGCs (**Figure 9C**). There was no current-response plateau despite increasing concentration of AP. AP concentrations greater than 1 μM were not tested due to neurosteroid ability to directly activate $GABA_A$ receptors at higher concentrations via a separate binding domain (Majewska et al., 1986; Wohlfarth et al., 2002).

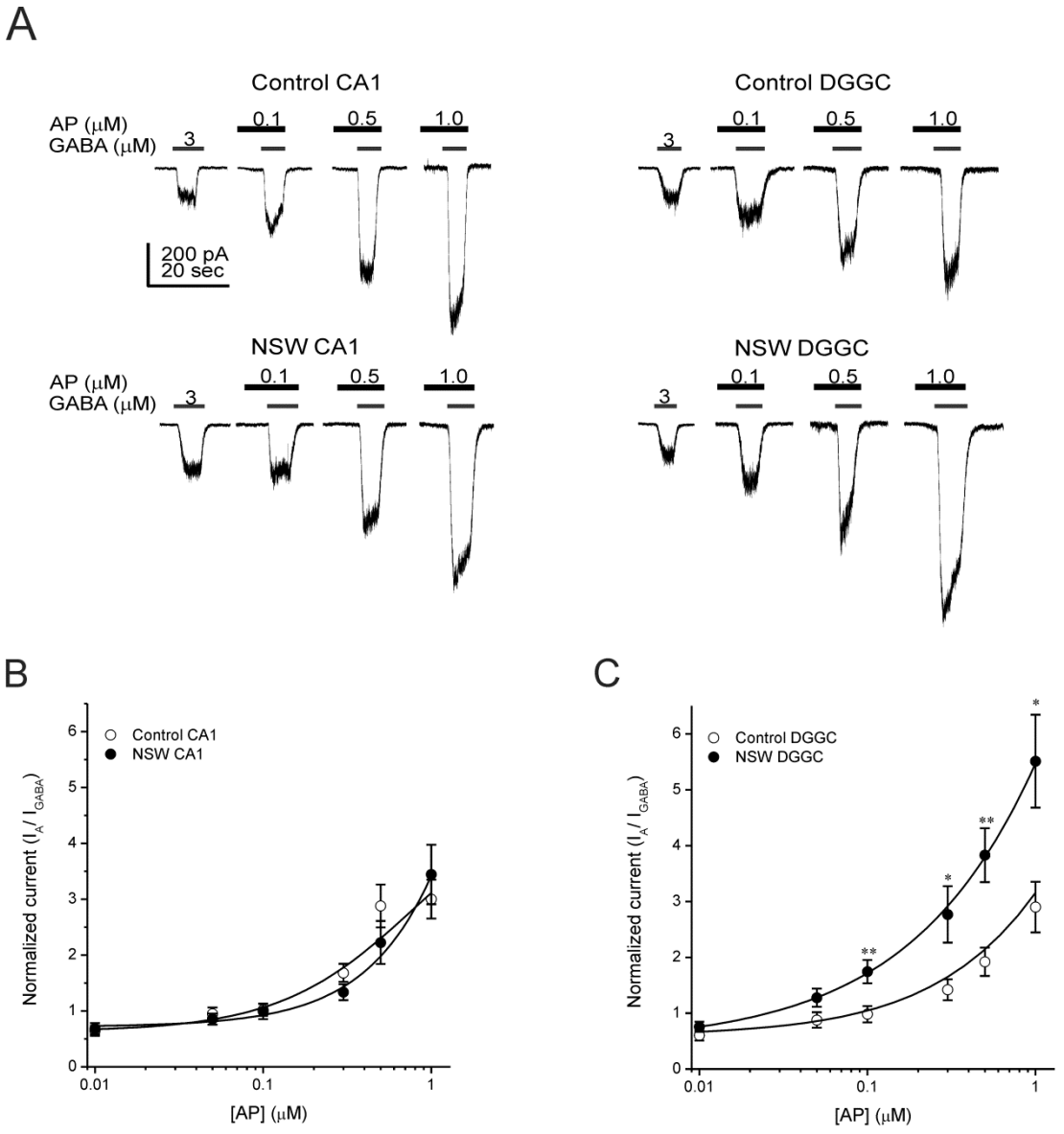


Figure 9. NSW enhances pharmacological sensitivity to allopregnanolone (AP) through allosteric potentiation of GABA-gated currents in dissociated DGGCs but not CA1 neurons. ***A***, Representative whole-cell current recordings of CA1 pyramidal cells and DGGCs in the NSW paradigm. Neurons displayed concentration-dependent responses to AP potentiation of 3 μM GABA (EC_{10}). ***B***, ***C***, Averaged concentration-response curves for fold-potentiation of AP (0.01 – 1.0 μM), normalized to baseline GABA EC_{10} (I_A/I_{GABA}). Potentiation was averaged for cells of similar condition and concentration following normalization to GABA response. NSW DGGCs displayed greater positive potentiation of GABAergic current by AP. Recordings in whole-cell mode, voltage clamped at -70 mV. * $p< 0.05$; ** $p< 0.01$ vs. control (n = 8-16 cells per condition and drug concentration).

IV.1.3 NSW confers enhanced extrasynaptic $GABA_A$ receptor-mediated tonic currents in DGGCs

The dentate gyrus up-regulation of δ-subunit in NSW is most functionally significant at extrasynaptic sites, where these receptor subunits preferentially localize and co-assemble on the membrane surface (Sun et al., 2004), with a denser staining in the molecular layer than the granule layer (Peng et al., 2002). To further confirm the functional significance of elevated levels of δ-subunit expression on extrasynaptic GABAergic network inhibition, we recorded GABA-gated tonic currents from WT DGGCs in a hippocampal slice preparation, in which synapses and dendritic connections remain functional. Bath perfusion of artificial cerebrospinal fluid contained 0.5 μM TTX to block action potentials. We examined tonic current modulation of GABA, THIP, and 1 μM GABA co-applied with AP. Gabazine (GZ, 50 μM) was applied to the slice perfusion to block phasic and tonic currents. Tonic current was measured as the shift in mean conductance before and after gabazine application. **Figure 10** depicts modulation of tonic current in granule cells during NSW. Endogenous tonic current was measured in the absence of GABA in the bath perfusion. Control DGGCs displayed significantly greater endogenous tonic current than NSW ($p = 0.0468$, n = 7-8 cells per group, **Figure 10C**). We subsequently measured tonic current with perfusion of 0.3 μM, 1 μM, or 3 μM GABA for a comparative profile of GABA binding of extrasynaptic receptors (**Figure 10C**). Both 0.3 ($p = 0.0398$) and 1 μM GABA ($p = 0.0480$) elicited greater tonic current in NSW than control DGGCs (n = 5-6 cells per group and concentration). In response to 1 μM GABA + 100 nM AP or 1 μM GABA + 300 nM AP, NSW DGGCs experienced significantly greater enhancement of tonic current than control DGGCs (100 nM AP: $p = 0.0088$; 300 nM AP: $p= 0.0167$; n = 8-10 cells per group, Figure **10C**).

We measured the root-mean-square (RMS) channel conductance during each drug application of current recording. Endogenous RMS noise was significantly greater in control (4.0 ± 0.2 pA) than NSW (3.4 ± 0.1 pA) tonic recordings ($p = 0.0274$, n = 6-7 cells per group), however 1 μM GABA RMS noise was not significantly different between NSW (3.9 ± 0.1 pA) and control (4.2 ± 0.2 pA; $p = 0.1567$; n = 8-12 cells per group). AP significantly increased the RMS noise compared to 1 μM GABA baseline, similar to our previous report (Wu et al., 2013). 300 nM AP modulated RMS noise to 5.6 ± 0.3 pA and 6.3 ± 0.4 pA in control and NSW DGGC, respectively, but this was not significantly different between conditions ($p = 0.1833$). Gabazine

application reduced the RMS noise in a withdrawal-dependent manner. Gabazine produced a greater reduction of AP-modulated RMS in NSW compared with control at 100 nM AP (Δ-0.9 ± 0.2 pA control vs. Δ-1.9 ± 0.2 pA NSW; $p = 0.0030$, n = 8-10 cells per group) as well as during 300 nM AP (Δ-2.3 ± 0.3 pA control vs. Δ-3.4 ± 0.3 pA NSW; $p = 0.0208$, n = 8 cells per group).

We explored THIP modulation of tonic current due to its high affinity and efficacy for δ-subunit extrasynaptic receptors (Mortensen et al., 2010; Meera et al., 2011) and different binding site than the allosteric site of neurosteroids (Brown et al., 2002; Stórustovu and Ebert, 2006). THIP was applied in an environment without exogenous GABA in the perfusion to allow for high $GABA_A$ receptor occupancy. A concentration-dependent response was examined for tonic current modulation by THIP in the range 0.03-1 μM, selective for δ-subunit binding (**Figure 10F**). Tonic currents of NSW DGGCs displayed significantly greater sensitivity to THIP at 0.1 and 0.3 μM concentrations than control DGGCs ($p < 0.05$, n = 4-6 cells per group and concentration). We observed desensitization of extrasynaptic current at 1 μM THIP (**Figure 10E**). While it is possible that 1 μM THIP is beyond δ-subunit selectivity, it was of note that extrasynaptic desensitization to THIP was more pronounced in NSW native DGGCs (Bright et al., 2011; Brown et al., 2002; Pandit et al., 2013). Previous studies describe that under steady-state conditions, THIP does not display super-agonist properties (Houston et al., 2012). Therefore, we measured peak tonic current 30 seconds after THIP administration, and we also measured tonic current at the time in which THIP-modulated conductance reached tonic desensitization. The criterion for determining the tonic desensitization is described in the Methods. Gabazine (50 μM) was applied after 2 minutes of recording to determine the total tonic current shift. We then compared the difference in peak and desensitized currents between control and NSW DGGCs (**Figure 10E-F**). The peak tonic current for 0.1 – 1 μM THIP was significantly different between control and NSW ($p = 0.0051$). NSW, but not control DGGCs displayed significant desensitization compared to the peak current induced by 1 μM THIP ($p = 0.0238$). Percent change of THIP-dependent current desensitization was significantly different between control (27.5 ± 5.5 %) and NSW DGGCs (50.8 ± 4.7 %; $p = 0.0229$, n = 5 cells per group). Together, these electrophysiology studies indicate neurosteroid withdrawal confers greater sensitivity of extrasynaptic currents to AP and the δ-specific agent THIP within dentate gyrus granule cells.

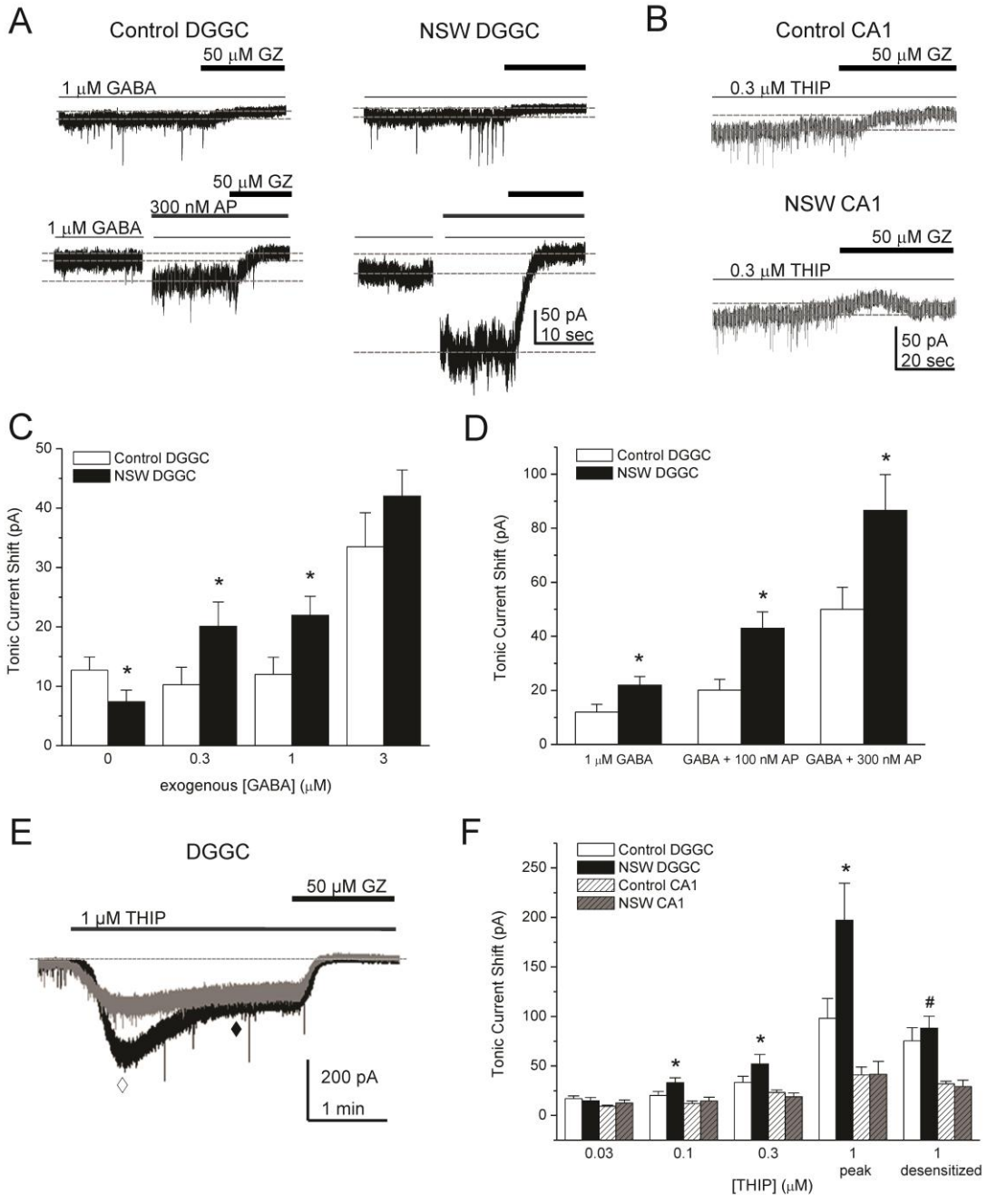


Figure 10. NSW enhances AP potentiation and THIP modulation of tonic currents in DGGCs in hippocampus slices.

Figure 10 Continued. ***A***, Representative GABAergic tonic current recordings from DGGCs. 300 nM AP produced a further negative shift in holding current level, and 50 µM of the competitive antagonist gabazine (GZ) produced a positive shift in holding level, blocking both phasic and tonic currents. Tonic current shift (pA) was measured as the total current change before and after gabazine application. The *first grey dotted line* in each raw trace represents the mean holding current during gabazine application. ***B***, Representative GABAergic tonic current recordings from CA1PCs in response to 0.3 µM THIP. ***C***, Concentration-response of DGGC tonic current to GABA. Endogenous tonic current (no exogenous GABA) shift was greater in control condition than NSW; however, NSW DGGCs displayed greater tonic current at 0.3 µM GABA and 1 µM GABA than control. ***D***, NSW DGGC tonic currents were more sensitive to modulation by AP than control at 100 nM and 300 nM AP potentiation of 1 µM GABA. ***E***, Representative tonic current recordings from control DGGC (*grey trace*) and NSW DGGC (*black trace*) due to 1 µM THIP application. ***F***, Quantification of tonic current during 0.03 to 1 µM THIP from CA1PCs and DGGC. 1 µM THIP administration at peak (◊ in Fig 5E) and desensitized (♦ in Fig 5E) tonic levels from DGGC were also compared. Whole-cell mode, voltage-clamp -65 mV. Data values represent mean ± S.E.M. *$p < 0.05$ vs. control DGGC current, #$p < 0.05$ vs. 1 µM THIP peak current, NSW (n=4 - 10 cells for each group and concentration).

IV.1.4 NSW does not confer enhanced extrasynaptic function in CA1 neurons

Previous models of NSW have suggested that increase to δ-subunit expression occurs in CA1 pyramidal cells (Smith et al., 2007; Sundstrom-Poromaa et al., 2002). There is a lack of definitive evidence that increases to δ-subunit expression within hippocampus promote functional, extrasynaptic current within CA1. Having demonstrated that DGGCs undergo functional increase in sensitivity to THIP after NSW, we examined tonic current modulation in CA1PCs. There were no significant differences to THIP-induced tonic currents in CA1 between control and NSW mice (**Figure 10F**). We did not observe significant desensitization of CA1 currents at 1 μM. This evidence further supports that δ-subunit expression up-regulation is functionally more significant to the granule cells of the dentate gyrus and not to CA1PCs.

IV.1.5 NSW does not confer enhanced AP sensitivity in δKO mice

To further confirm whether NSW-induced increase in neurosteroid sensitivity is due to extrasynaptic δ-subunit $GABA_A$ receptors, we utilized knockout mice that lack expression of δ-containing receptors in the brain as a robust genetic model. Neurons were acutely dissociated from δKO female mice treated in the perimenstrual-like withdrawal paradigm. Voltage-clamp recordings were obtained from DGGC neurons in response to AP co-applied with 3 μM GABA (**Figure 11A**), similar to the experiments performed in Figure 9. A concentration-response curve was derived for AP potentiation of GABAergic current (**Figure 11B**), and fractional current responses of AP were compared between NSW and vehicle-injected control δKO animals. In δKO granule cells, AP (0.05-0.30 μM) and GABA produced less modulation of $GABA_A$ receptor currents than that of GABA alone. NSW and control DGGCs from δKO mice displayed no significant differences at any of the concentrations tested (n = 8-12 cells per each group and drug concentration). In addition, modulation by AP was significantly lower in δKO DGGCs than in WT DGGCs.

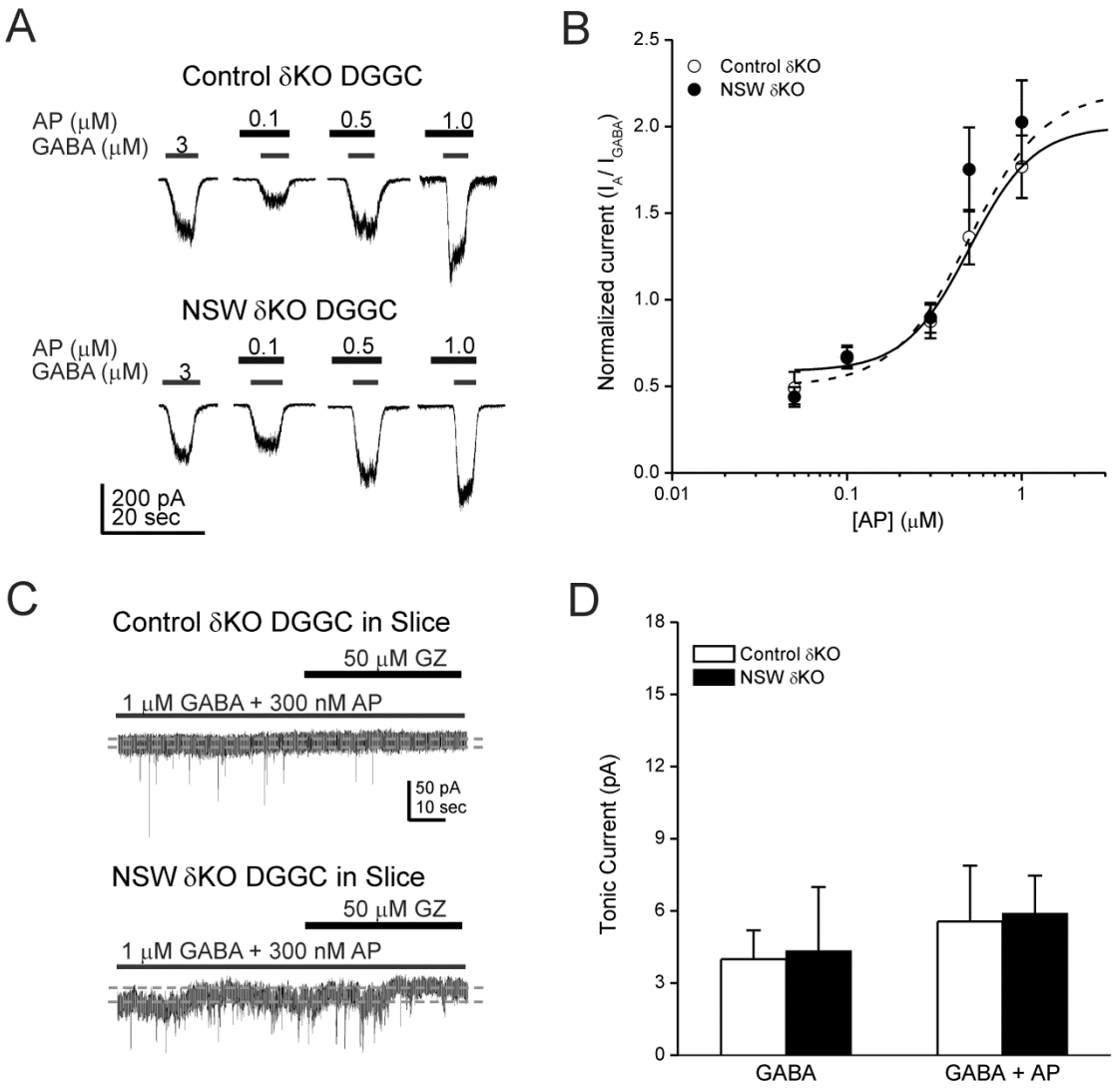


Figure 11. AP sensitivity is attenuated in δKO mice undergoing NSW. ***A***, Representative whole-cell current recordings of dissociated DGGCs from δKO mice. ***B***, Concentration-response curve of δKO DGGCs due to AP modulation of GABA-gated current (I_A/I_{GABA}). AP potentiation was not significantly different between control and NSW DGGCs at any of the AP concentration tested (0.05 – 1 μM). ***C***, Tonic current recordings from control and NSW DGGCs in hippocampus slices from δKO mice. The *grey dotted line* represents the mean holding current during gabazine (GZ) application. 50 μM gabazine antagonized phasic $GABA_A$ receptor currents but caused little to no shift in the tonic current holding level of δKO neurons. ***D***, Tonic current shift due to gabazine is reduced and minimal in δKO DGGCs in control and NSW. There were no significant differences in tonic current between NSW and control DGGCs during 1 μM GABA or during 1 μM GABA + 300 nM AP application. Data represent mean ± S.E.M. (n= 5-12 cells). Whole-cell mode, voltage clamped at -70 mV (***A***-***B***) or -65 mV (***C***-***D***).

We acquired tonic current recordings from δKO DGGCs to ascertain the degree of AP sensitivity in a hippocampal network lacking δ-subunit (**Figure 11C-D**). Changes to tonic current, RMS, and enhanced AP sensitivity were not evident in δ-subunit knockout animals subjected to withdrawal. Tonic currents were highly attenuated, and control and NSW knockouts displayed no significant differences in 1 μM GABA ($p = 0.8860$; $n = 5$ cells per group) or 1 μM GABA and 300 nM AP ($p = 0.9064$; $n = 6$ cells per group). Overall, these results indicate a lack of NSW-induced function in δ-subunit knockout animals. The neurosteroid sensitivity present in withdrawn mice is attenuated when the δ-subunit is genetically and functionally removed from the hippocampus, disallowing plastic increase in δ expression.

IV.1.6 NSW influences synaptic $GABA_A$ receptor mIPSC amplitude and kinetics

To determine the extent of withdrawal-induced modulation by allopregnanolone on synaptic receptors, GABAergic miniature IPSCs were recorded in DGGCs from brain slices in the presence of 0.5 μM TTX in perfusion and 5 mM QX-314 in the recording electrode pipette. The properties of amplitude, 10-90% rise-time, decay time constants, and inter-event interval (IEI, as a measure of frequency) were analyzed from mIPSCs (**Table 2 and Figure 12**). The average mIPSC from each neuron was best fit with a double-exponential decay curve, depicted as τ_1 and τ_2. A weighted decay constant, τ_w was also determined (see Methods section III.4). mIPSC frequency is related to functional number of synapse release sites and the rate of presynaptic release (Cherubini and Conti, 2001). $GABA_A$ receptor channel kinetics are highly influenced by receptor subunit composition (Carver and Reddy, 2013; Smith et al., 2007). Postsynaptic receptor density, synaptic vesicle GABA content, and dendritic cable properties contribute to mIPSC amplitude (Cherubini and Conti, 2001; Nusser et al., 1997). We hypothesized that NSW would not alter presynaptic GABA release properties; however, we proposed that NSW-induced sensitivity of allopregnanolone may affect postsynaptic current and kinetics due to the α_4 and δ subunit expressional changes (**Figure 12**). Within recordings of endogenous (no GABA) or 1 μM GABA perfusion, the amplitude, kinetics, and frequency were not significantly different between withdrawal and control DGGCs (**Table 2**). These experiments were repeated with 1 μM TTX to ensure the recorded IPSCs were action-potential-independent. There were no significant differences in amplitude, decay constants, and frequency of mIPSCs in comparison of 0.5 μM and 1.0 μM TTX. In the addition of 300 nM AP, peak amplitude was significantly greater in

withdrawal than control DGGCs ($p = 0.0046$; $n = 5$ cells per group). Neurosteroids such as AP increase the channel opening probability of $GABA_A$ receptors and prolong the decay kinetics (Carver and Reddy, 2013). The τ_w constant derived from τ_1 and τ_2 time constants of phasic $GABA_A$ receptors from DGGCs was consistent with previous reports (Sun et al., 2007). AP (300 nM) resulted in a significant increase in τ_2 and τ_w decay time constants of both control and NSW ($p = 0.0011$ vs. control 1 μM GABA; $p < 0.0001$ vs. NSW 1 μM GABA). Whereas the control mIPSCs exhibited an average 62.1 ± 6.0 % increase to τ_2 decay upon AP modulation, the NSW mIPSCs exhibited an average 135.8 ± 5.2 % increase to τ_2 decay. Control mIPSCs had an average of 33.1 ± 5.5 % increase to τ_w decay upon AP modulation, and NSW mIPSCs had an average 66.8 ± 6.3 % increase to τ_w decay. This difference in mean percent change of τ_2 and τ_w was statistically significant between control and NSW groups ($p < 0.0001$ for τ_2, $p = 0.0008$ for τ_w). Data represent mean values for 5-10 cells per group and application.

In order to determine if the NSW-induced changes to synaptic activity we observed are related to δ-subunit expression, we recorded mIPSCs from δ-knockout mice subjected to the withdrawal paradigm. AP did not significantly prolong the decay in δKOs as that occurred in WT neurons, and there were no significant differences in mIPSC properties between control and withdrawal in knockout DGGCs (**Table 2**). In addition, during AP administration, δKO mIPSCs displayed faster τ_2 and τ_w decay than WT mIPSCs in both control and withdrawal conditions.

Our findings indicate that δ-containing receptors mediate the prolongation of mIPSC decay in the presence of AP. NSW-induced change to phasic decay kinetics did not occur with 1 μM GABA alone. However, NSW tonic currents displayed greater sensitivity to 1 μM GABA. This difference in activity may represent distinct receptor subtype compositions and their GABA-gating properties within the native neuronal population (Mortensen et al., 2010). We demonstrated that AP is able to alter the efficacy state and channel opening of the phasic-responding receptors in withdrawn animals above control conditions (Bianchi and Macdonald, 2003). It is possible that a perisynaptic population of $GABA_A$ receptors could respond to synaptic spillover of GABA during up-regulated δ expression. However, there is evidence that suggests δ-containing receptors are relatively insensitive to phasic GABA activation that could occur through synaptic spillover (Bright et al., 2011).

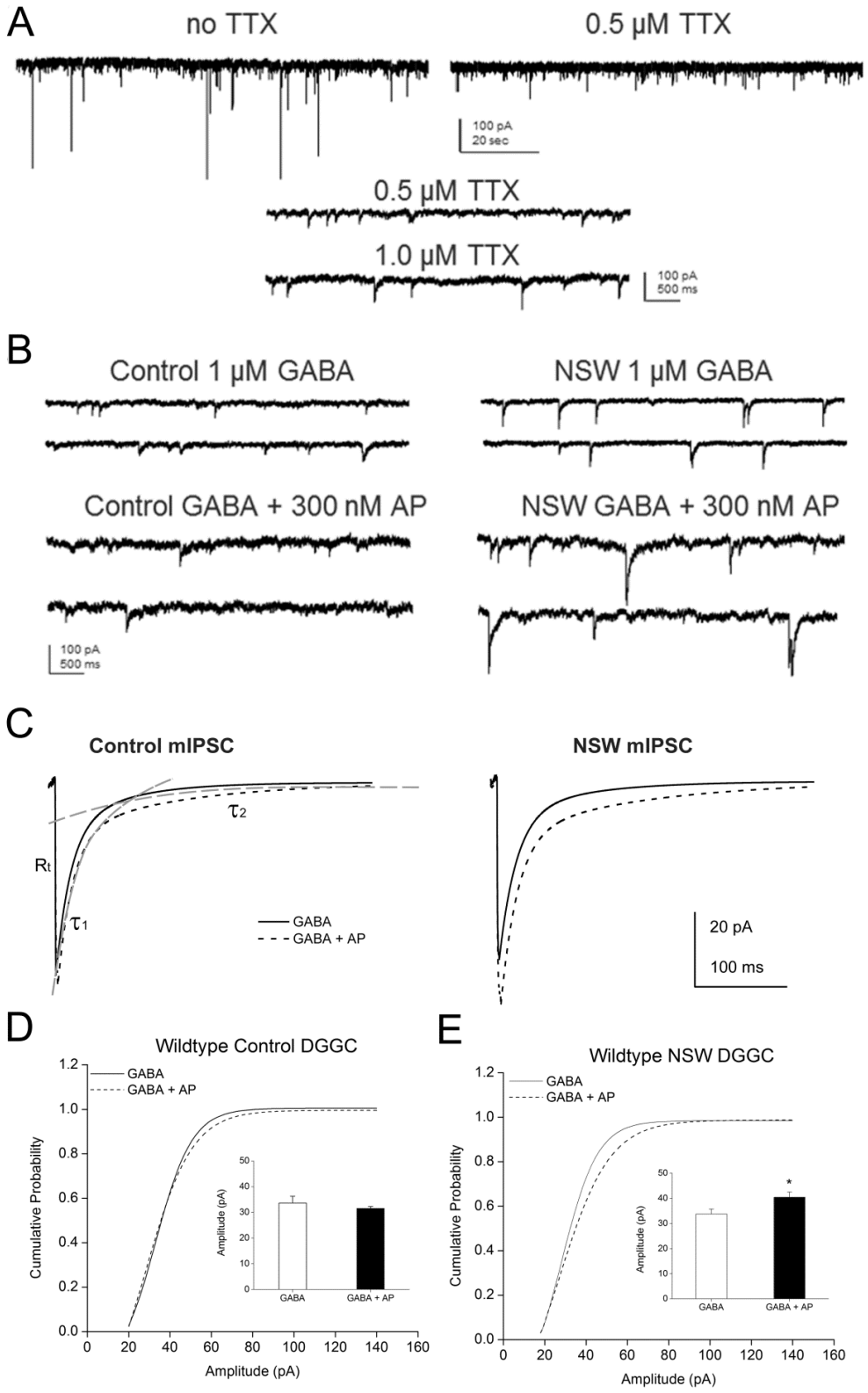


Figure 12. mIPSCs are sensitive to AP in mice undergoing NSW.

Figure 12 Continued. Phasic events were recorded from patch-clamped DGGCs in hippocampus slice. $GABA_A$ receptor synaptic activity was isolated using TTX, APV, and DNQX. Synaptic current was blocked with gabazine (50 µM). ***A***, Representative traces of IPSCs before and after TTX application and mIPSCs in 0.5 µM or 1.0 µM TTX. ***B***, mIPSC recordings during GABA and AP modulation. RMS channel noise increased during AP application. ***C***, Averaged mIPSC events recorded from control and NSW DGGCs in the presence of 1 µM GABA (*solid line*) or 1 µM GABA co-applied with 300 nM AP (*dashed line*), and bi-exponential fitted for fast and slow decay time constants, τ_1 and τ_2 (gray dash lines), respectively. NSW DGGCs displayed significantly greater amplitude ($p = 0.0046$) and τ_2 decay ($p = 0.0011$) during application of 300 nM AP than control DGGCs (n = 5-10 cells per group) (R_t: downward rise time. see Table 2). Cumulative probability curves for wildtype control (***D***) and NSW (***E***) mIPSC amplitude, plotted from all events. Kolmogorov-Smirnov test was used to compare mIPSC before and after application of AP in DGGCs (n = 5-10 per group). ***D-E inlay***, Mean peak amplitude of mIPSCs. * $p < 0.05$. Recordings whole-cell mode, voltage-clamp -65 mV.

Table 2. $GABA_A$ receptor-mediated mIPSC characteristics in control and NSW mice

Group (n)	Amplitude (pA)	$Rise_{10-90\%}$ (ms)[1]	Decay τ_1 (ms)[1]	Decay τ_2 (ms)[1]	Decay τ_w (ms)	IEI (s)[2]
WT Control endogenous (5) 0.5 μM TTX	30.8 ± 1.6	1.2 ± 0.2	12.6 ± 0.8	51.6 ± 3.9	27.2 ± 1.5	1.4 ± 0.1
WT Control, endogenous (5) 1.0 μM TTX	27.3 ± 2.4	1.1 ± 0.2	10.2 ± 0.6	48.5 ± 5.8	25.2 ± 2.9	1.6 ± 0.1
WT NSW endogenous (5) 0.5 μM TTX	30.3 ± 1.9	1.0 ± 0.1	11.5 ± 1.3	49.2 ± 5.5	25.3 ± 1.1	1.1 ± 0.2
WT NSW, endogenous (5) 1.0 μM TTX	32.7 ± 1.4	0.9 ± 0.1	13.9 ± 1.1	49.0 ± 2.3	26.4 ± 2.8	1.4 ± 0.1
WT Control: +1 μM GABA (10)	33.7 ± 2.6	1.2 ± 0.3	12.4 ± 0.6	49.6 ± 3.3	26.0 ± 0.9	1.6 ± 0.3
WT NSW: + 1 μM GABA (10)	33.8 ± 2.0	1.2 ± 0.2	14.4 ± 1.4	43.3 ± 4.2	25.3 ± 1.9	1.3 ± 0.2
δKO Control: + 1 μM GABA (9)	33.7 ± 2.2	1.3 ± 0.1	12.2 ± 0.9	47.8 ± 4.0	26.0 ± 2.6	1.8 ± 0.1
δKO NSW: + 1 μM GABA (6)	32.4 ± 2.3	1.1 ± 0.2	11.7 ± 1.8	45.8 ± 6.0	22.5 ± 2.5	1.8 ± 0.6
WT Control: + GABA+300 nM AP (5)	31.5 ± 0.8	1.4 ± 0.1	12.6 ± 1.8	*80.4 ± 8.3	*34.6 ± 1.4	1.7 ± 0.2
WT NSW: + GABA +300 nM AP (5)	*#40.5 ± 2.1	1.6 ± 0.3	16.5 ± 2.0	*102.1 ± 7.9	*42.2 ± 3.1	1.6 ± 0.3
δKO Control: +GABA +300 nM AP(9)	34.8 ± 1.3	1.5 ± 0.2	15.3 ± 1.4	&58.5 ± 5.1	&29.5 ± 1.1	1.7 ± 0.3
δKO NSW: + GABA +300 nM AP(5)	&34.9 ± 2.0	1.0 ± 0.1	10.1 ± 1.0	&47.9 ± 7.5	&23.6 ± 2.3	1.6 ± 0.4

mIPSCs were recorded in the presence of 0.5 μM TTX unless otherwise denoted. Groups denoted as + GABA + AP were recorded in the presence of 1 μM GABA and 300 nM AP. Recordings were from voltage clamped (-65 mV) dentate gyrus granule cells in hippocampus slices.

[1]Rise time, decay τ_1, and decay τ_2 are represented as R_t, τ_1, and τ_2 in Fig 7C. $Rise_{10-90\%}$ represents as duration for downward shift 10 to 90% of the amplitude. [2]IEI = Inter-event interval as a measure of frequency.

* $p < 0.05$ vs. WT NSW + 1 μM GABA alone; # $p < 0.05$ vs. WT Control + GABA +300 nM AP; & $p < 0.05$ vs. WT NSW + GABA +300 nM AP; independent two-tailed t-test.

Since low, ambient GABA (1 μM) was unable to elicit a significant difference in the phasic current, we explored mIPSCs with higher concentrations of exogenous GABA to engender a condition of higher efficacy synaptic response in both WT and δKO DGGCs (**Table 3**). GABA concentrations of 3 and 10 μM were sufficient to significantly increase both the amplitude, τ_2 and τ_w decay of mIPSCs from WT control DGGCs (3 μM GABA: amplitude: 44.0 ± 3.0 pA, τ_1: 15.4 ± 2.2 ms τ_2: 70.9 ± 3.8 ms, τ_w: 34.9 ± 2.3 ms; 10 μM GABA: amplitude: 54.1 ± 5.7 pA, τ_1: 16.5 ± 2.7 ms, τ_2: 109.8 ± 12.0 ms, τ_w: 45.7 ± 6.6 ms; n = 5-6 cells per concentration). Compared with 1 μM GABA, WT DGGCs displayed an average 42.9 ± 2.6 % increase in τ_2 in 3 μM GABA, whereas DGGCs in 10 μM GABA had an average 121.4 ± 10.2 % increase in τ_2. We also explored mIPSCs within δKO DGGCs in response to 3 and 10 μM GABA (3 μM GABA: amplitude: 30.2 ± 1.0 pA, τ_1: 18.6 ± 1.7 ms τ_2: 57.8 ± 3.1 ms, τ_w: 32.4 ± 1.5 ms; 10 μM GABA: amplitude: 48.9 ± 6.8 pA, τ_1: 15.7 ± 1.5 ms, τ_2: 64.8 ± 5.8 ms, τ_w: 34.3 ± 1.0 ms; n = 4-5 cells per concentration). δKO DGGCs had an average 20.9 ± 1.4 % change in τ_2 in 3 μM GABA and an average 35.6 ± 3.1 % change in τ_2 in response to 10 μM GABA when compared with 1 μM GABA. The difference in τ_2 decay was significantly reduced in δKO DGGCs compared to WT for each GABA concentration ($p < 0.05$), denoting an increased contribution of δ-subunit to phasic currents in control conditions, albeit at supraphysiological concentrations of GABA. Our data provide pharmacologically significant evidence that δ-containing receptors require higher-efficacy gating in order to mediate phasic currents, such as that occurring by neurosteroid potentiation of GABA (Bianchi and Macdonald, 2003). In our model, neurosteroid withdrawal is sufficient to promote prolongation of post-synaptic current decay and amplitude in response to AP, but not to low (≤ 1 μM), ambient GABA alone.

Interestingly, 3 and 10 μM GABA significantly decreased mIPSC frequency, a measure of presynaptic GABA release when bath applied to hippocampal slices (**Table 3**). This suggests localized presynaptic depression of GABAergic terminals synapsing to DGGCs in high concentrations of extracellular GABA, both in WT and δKO conditions. Another mechanism for lower mIPSC frequency could be that the higher receptor occupancy or continual activation at greater GABA concentrations results in reduced GABA gating by a diminished receptor pool. AP (0.3 μM) modulation of mIPSCs displayed a trend of decreased frequency, however it was not statistically significant (**Table 2**).

Table 3. $GABA_A$ receptor-mediated mIPSC characteristics in WT and δKO mice

Group (n)	Amplitude (pA)	$Rise_{10-90\%}$ (ms)[1]	Decay τ_1 (ms)[1]	Decay τ_2 (ms)[1]	Decay τ_w (ms)	IEI (s)[2]
WT: +1 μM GABA (10)	33.7 ± 2.6	1.2 ± 0.3	12.4 ± 0.6	49.6 ± 3.3	26.0 ± 0.9	1.6 ± 0.3
WT: +3 μM GABA (6)	*44.0 ± 3.0	1.6 ± 0.4	15.4 ± 2.2	**70.9 ± 3.8	**34.9 ± 2.3	*2.6 ± 0.1
WT: +10 μM GABA (5)	*54.1 ± 5.7	*2.2 ± 0.5	16.5 ± 2.7	**109.8 ± 12.0	**45.7 ± 6.6	*2.8 ± 0.2
δKO: + 1 μM GABA (9)	33.7 ± 2.2	1.3 ± 0.1	12.2 ± 0.9	47.8 ± 4.0	26.0 ± 2.6	1.8 ± 0.1
δKO: + 3 μM GABA (4)	30.2 ± 1.0	1.5 ± 0.6	#18.6 ± 1.7	&57.8 ± 3.1	32.4 ± 1.5	#3.1 ± 0.2
δKO: + 10 μM GABA (5)	#48.9 ± 6.8	1.8 ± 0.3	15.7 ± 1.5	#†64.8 ± 5.8	34.3 ± 1.0	#3.1 ± 0.7

mIPSCs were recorded in the presence of 0.5 μM TTX. Groups denoted as WT were recorded from WT female mice whereas δKO denotes recordings from δ-subunit knockout female mice. Recordings were from voltage clamped (-65 mV) dentate gyrus granule cells in hippocampus slices.

[1]Rise time, decay τ_1, and decay τ_2 are represented as R_t, $\tau_{1, \text{ and }} \tau_2$, similar to that of Fig 7C. $Rise_{10-90\%}$ represents as duration for downward shift 10 to 90% of the amplitude. [2]IEI = Inter-event interval as a measure of frequency.

* $p < 0.05$ vs. WT: +1 μM GABA ; ** $p < 0.01$ vs. WT: +1 μM GABA ; # $p < 0.05$ vs. δKO: +1 μM GABA ; & $p < 0.05$ vs. WT: +3 μM GABA; † $p < 0.05$ vs. WT: +10 μM GABA; independent two-tailed t-test.

IV.1.7 NSW causes increased susceptibility to hippocampus kindling seizures

To investigate whether the NSW-induced changes in $GABA_A$ receptors mediating tonic inhibition affects neuronal network excitability in a seizure model, we studied the susceptibility of neurosteroid withdrawn mice to seizures in rapid hippocampus kindling (Reddy and Mohan, 2011; Wu et al., 2013). Adult female mice were surgically implanted with a bipolar electrode in the hippocampus. Following surgery recovery and subsequent withdrawal paradigm, the mice were subjected to rapid kindling stimulations at 125% AD (afterdischarge) threshold at 30 minute intervals until stage 5 seizures were consistently reached. The progression of rate of kindling, electrographic AD threshold, and cumulative AD activity time for kindling criterion were recorded as main indices of epileptogenesis (**Figure 13**). Mean rate of kindling was significantly faster in NSW animals (**Figure 13B**, $p = 0.0098$). Mean AD threshold current was significantly reduced in NSW mice (**Figure 13C**, $p = 0.0149$). Cumulative AD duration was not significantly different between control and withdrawal groups (**Figure 13D**, $p = 0.1384$). The amplitude of AD spike activity was markedly greater in NSW mice than in control mice (data not shown). The rapid kindling experiments were conducted in δKO mice with and without NSW induction. The δKO animals did not display significant differences in any of the seizure parameters between control and NSW groups (**Figure 13B-D**). Thus, these results are consistent with the increased excitability and susceptibility of animals undergoing NSW with δ-subunit intact, which is a key triggering factor for catamenial seizure exacerbation.

IV.1.8 Fully-kindled, NSW mice exhibit increased sensitivity to antiseizure effects of AP

To determine the therapeutic potential of neurosteroids in perimenstrual catamenial epilepsy, we tested the effect of neurosteroid AP on heightened seizures caused by perimenstrual-like neurosteroid withdrawal in fully-kindled mice exhibiting stage 5 seizures. Female WT and δKO mice were subjected to once-daily kindling until they exhibited stage 5 seizures for 3 consecutive days, which is considered the fully kindled state. WT mice reached the fully kindled state with consistent stage 5 seizures after 10 to 16 stimulations (**Figure 13B**), as reported previously (Reddy et al., 2012). Fully-kindled WT and δKO mice undergoing NSW condition were treated with AP or vehicle injections. Fifteen minutes after AP administration, animals were stimulated at their designated AD threshold. Behavioral seizure stage and electrographic AD duration were

evaluated as criteria of drug efficacy for sensitivity assessment (**Figure 14**). AP (1-10 mg/kg, sc) exerted dose-dependent suppression of the behavioral seizures (**Figure 14A**) and AD duration (**Figure 14B**) in WT control (non-withdrawal) mice. In contrast, after neurosteroid withdrawal, AP produced greater suppression of behavioral seizures and AD duration in WT mice with significant effects at 1 and 5 mg/kg compared with the control group ($p < 0.01$, n = 6-9 mice per group). These experiments were repeated in fully-kindled δKO mice. In contrast to WT counterparts, fully-kindled δKO mice undergoing NSW failed to exhibit any significant difference in seizure expression (**Figure 14C**) and AD duration (**Figure 14D**) following AP (1-10 mg/kg, sc) treatment (n = 6-9 mice per group). These results confirm enhanced neurosteroid sensitivity in WT mice due to up-regulation of δ-subunit. However, the time-course profiles for onset and duration of seizure expression following AP (10 mg/kg) treatment were similar in WT and δKO mice (**Figure 14E-F**), indicating the lack of significant pharmacokinetic factors in drug sensitivity related to genotype. Moreover, plasma levels of AP achieved at various doses of AP treatment (10 mg/kg) were correlated with seizure protection in WT control and withdrawn groups (**Figure 14E-F**), indicating a linear pharmacokinetic-pharmacodynamic relationship. Overall, the neurosteroid AP produced enhanced antiseizure efficacy (50%) in WT NSW but not δKO NSW animals, confirming the δ-subunit related enhanced sensitivity to neurosteroids in the neurosteroid-withdrawal model of catamenial epilepsy (Reddy and Rogawski, 2001; Reddy et al., 2012).

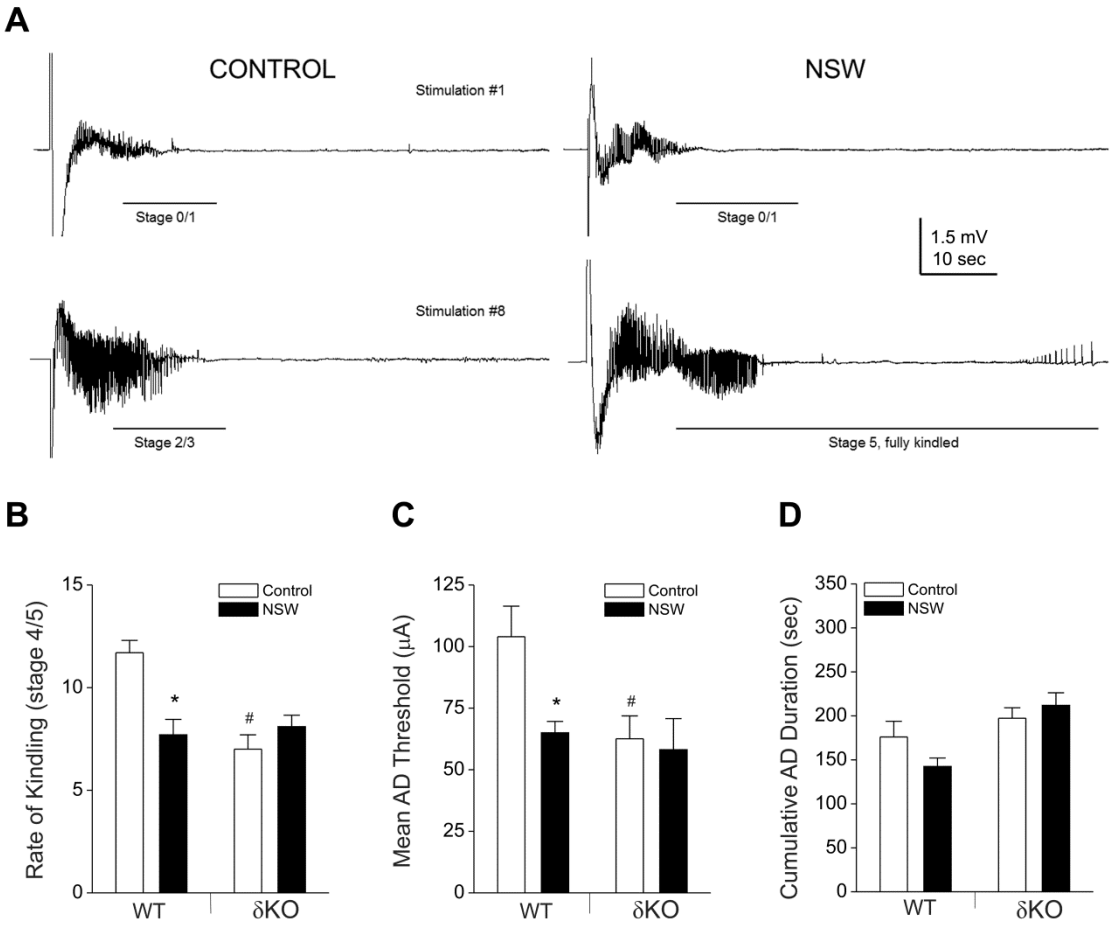


Figure 13. Changes in susceptibility to hippocampus kindling epileptogenesis in NSW WT and δKO mice. Adult, female mice in the withdrawal paradigm were subjected to rapid kindling stimulations at 125% afterdischarge (AD) threshold at 30 minute intervals until consistent display of stage 5 seizures. ***A***, Representative electrographic AD seizure activity recorded immediately after kindling stimulation of the hippocampus, recorded in control and NSW wildtype mice. The black bar above the stage designation denotes the period of the electrograph in which the animal experienced seizure activity. ***B***, WT mice experiencing NSW developed stage 4/5 seizures in significantly less number of stimulations than control mice, as denoted by rate of kindling. ***C***, WT mice undergoing NSW mice showed a lower mean AD threshold than control mice, requiring less current stimulation to achieve epileptogenic seizure activity. Control δKO mice exhibited a more rapid kindling and had lower AD threshold than WT control counterparts. ***D***, Cumulative AD duration was similar between control and NSW groups. * $p < 0.05$ vs. control of similar genotype, # $p < 0.05$ vs. WT control (n = 9-15 animals per group).

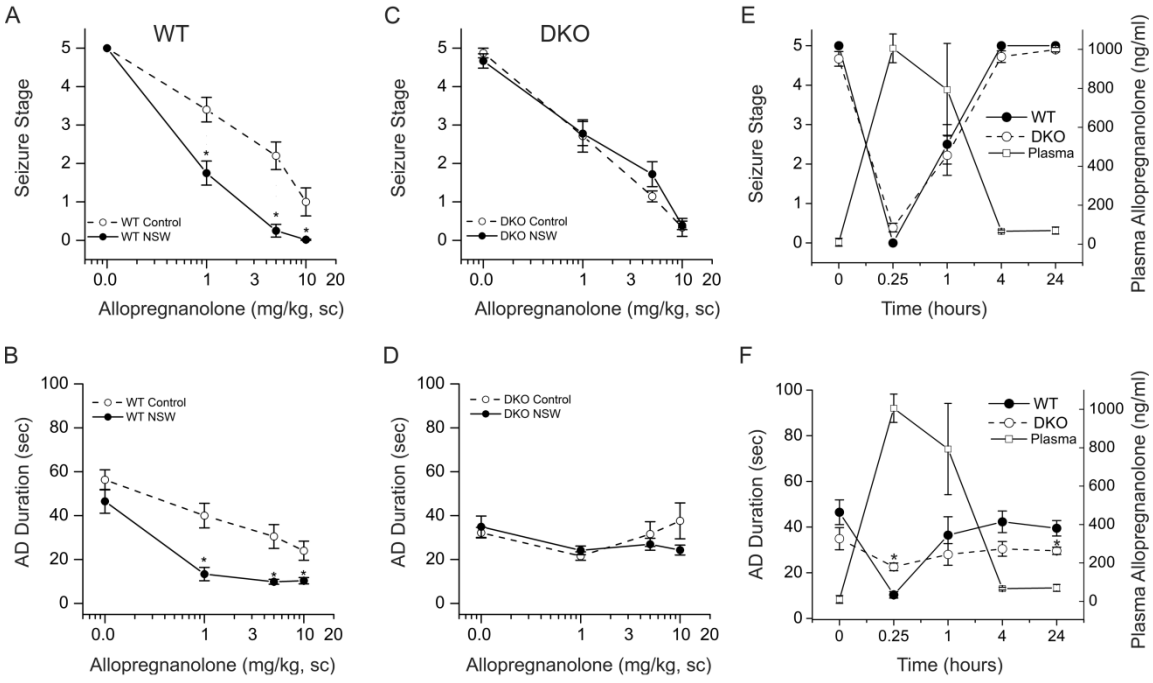


Figure 14. NSW confers antiseizure effects to AP in fully kindled WT but not δKO mice.
Fully kindled mice undergoing NSW were utilized for testing the effect of allopregnanolone on seizure activity. ***A***, Dose-response curves for AP-induced (1-10 mg/kg, s.c.) suppression of behavioral seizure stage and ***B***, AD duration in WT mice. ***C***, Dose-response curves for AP-induced (1-10 mg/kg, s.c.) suppression of behavioral seizure stage and ***D***, AD duration in δKO mice. There were no significant differences between δKO control and NSW seizure modification by AP. ***E***, Time-course correlation between plasma AP levels and seizure stage, and ***F***, AD duration in WT and δKO mice. Vehicle or AP was injected 15 min before kindling stimulations or plasma sample collection. (***A-D***) * $p < 0.05$ vs. control. (***F***) * $p < 0.05$ vs. WT (n = 6-9 animals per group).

IV.2 Structure-activity Relationship of Neurosteroid Modulation of δ-containing Extrasynaptic $GABA_A$ Receptor-mediated Tonic inhibition in the Hippocampus DGGCs

IV.2.1 GABA-gated whole-cell currents in acutely dissociated DGGCs in germline δKO mice

To determine the impact of δ-subunit on GABA-gated current, we evaluated whole-cell GABA concentration-responses from acutely dissociated DGGCs in WT and δKO mice. Native DGGCs of δKO mice exhibited significantly greater GABA potency than WT (**Figure 15A**). WT DGGCs had an average GABA EC_{50} of 28.2 ± 0.9 μM and a Hill coefficient of 1.35 ± 0.04 (n = 11), whereas δKO DGGCs had an EC_{50} of 15.7 ± 0.7 μM and a Hill coefficient of 1.21 ± 0.02 (n = 7). All cells displayed $GABA_A$ receptor current saturation at 1 mM GABA. From the same whole-cell currents, we measured the percent desensitization of the $GABA_A$ chloride channel macrocurrents subsequent to barrel-pipette application of GABA. In the granule cells, 0.1–10 μM concentrations of GABA displayed little or no desensitization upon application. However, 100 – 1000 μM GABA displayed peak current responses that rapidly desensitized and were curve fitted by the Chebyshev method with a single-exponent decay function. We observed that δKO DGGC macro-currents had greater extent of desensitization than WT DGGCs due to GABA in the range of 100 - 1000 μM GABA (**Figure 15B**). In addition, the average 10-90% decay time for macro-currents in saturating 1 mM GABA was significantly greater for WT DGGCs (7.53 ± 0.40 s) than δKO DGGCs (4.60 ± 0.50 s; p = 0.0002; n = 7-10 samples per group).

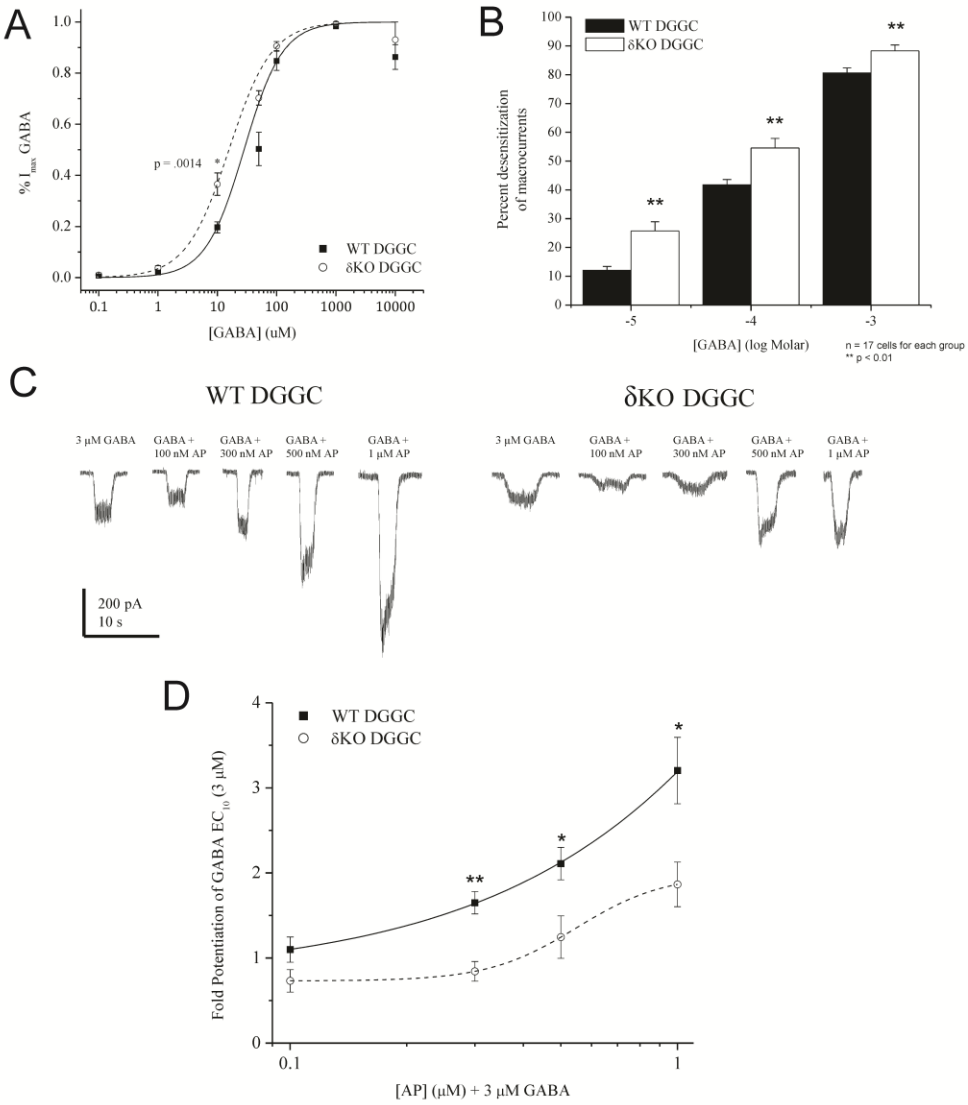


Figure 15. Reduced GABAergic inhibition from dissociated DGGCs in δKO mice by patch-clamp recordings. ***A***, GABA concentration-repsonse curve of dissociated WT and δKO DGGCs. Each data point was fit according to the maximum GABA-gated current at the saturating concentration (1000 μM). EC_{50} and Hill coefficient values are noted in the text. At 10 μM GABA, δKO responses were significantly greater than WT. ***B***, Percent desensitization of GABAergic macrocurrent kinetics due to rapid application of 10 (-5 log Molar), 100 (-4 log Molar), or 1000 (-3 log Molar) μM GABA. δKO cells had significantly greater percent desensitization than WT. ***C***, Representative traces of dissociated DGGC response to potentiation of 3 μM GABA (EC_{10}) by AP in WT and δKO DGGC. ***D***, Concentration-response quantification of fold-potentiation by AP. δKO cells had significantly reduced response to AP compared with WT. n = 8-10 cells per group. * $p < 0.05$, ** $p < 0.01$ by unpaired t-test. All cells dissociated from diestrus, WT female mice dentate gyrus. Recordings in whole-cell mode, voltage-clamp -70 mV.

IV.2.2 Diminished allosteric potentiation by AP in dissociated δKO DGGCs

Pharmacological response to the AP potentiation of $GABA_A$ receptors was obtained in whole-cell current recordings from dissociated DGGCs. GABA EC_{10} (3 μM) was bath applied to determine control current. Subsequently, increasing concentrations of AP were co-applied with GABA to achieve an allosteric concentration-response (**Figure 15C**). Fractional potentiation of GABAergic currents by AP is represented by mean fold-enhancement of the GABA EC_{10} response. AP enhanced whole-cell current in a concentration-dependent manner in WT DGGCs. AP (0.1 – 1 μM) did not significantly potentiate currents in the absence of GABA, signifying a lack of direct activation of receptors at submicromolar concentrations in these cells. Thus, these responses represent an allosteric modulation by AP at the $GABA_A$ receptors. δKO cells exhibited significantly reduced AP potentiation of GABA current than WT (**Figure 15D**). At 0.3, 0.5, and 1 μM AP, WT cells displayed enhanced GABA currents significantly greater than δKO counterparts. In addition, within δKO cells, only the 1 μM AP response elicited potentiation significantly above the 3 μM GABA control response. AP (1 μM) potentiated an average 3.20-fold of current in WT DGGCs, whereas δKO DGGCs produced 1.87-fold of the GABA EC_{10} response.

IV.2.3 Neurosteroid structure-activity relationship on extrasynaptic $GABA_AR$-mediated tonic inhibition

GABA-gated responses on tonic inhibition

To obtain extrasynaptic $GABA_A$ receptor responses and derive a basal I_{tonic} prior to neurosteroid exposure, we examined exogenous GABA in patch-clamp recordings from DGGCs in the hippocampus slice preparation. Following GABA perfusion, the competitive antagonist gabazine (50 μM) was applied to block all $GABA_A$ receptor-mediated current. The difference between holding current level before and after gabazine-induced blockade was determined as the tonic current shift (see Methods section III.4). The current shift was normalized in each neuron to the membrane capacitance as measure of current density (pA/pF), represented as I_{tonic}. In WT DGGCs, GABA increased tonic current in a concentration-dependent manner (**Figure 16**). However, the average current was not significantly different between 0.3 μM and 1 μM GABA, which is within the physiological range of extracellular GABA (Lerma et al., 1986). A small degree of tonic current desensitization was observed at 10 μM, but not at lower concentrations. In contrast to WT controls, δKO exhibited significantly attenuated I_{tonic} levels to GABA (0.1–3 μM). GABA tonic currents of δKO cells in this range were not significantly different than the response without exogenous GABA. Only 10 μM GABA significantly increased current in δKO above the endogenous condition ($p = 0.0092$). In the presence of 10 μM GABA, tonic current was not significantly different between WT and δKO DGGC ($p = 0.1260$), signifying GABA-gating of non-δ- (or γ_2-) containing receptors.

The criterion for determining the δ-containing, extrasynaptic EC_{100} was the highest GABA concentration at which tonic current in δKO DGGCs was not significantly different than the condition of no exogenous GABA. Therefore, EC_{100} denotes the GABA concentration at which synaptic non-δ-containing receptors do not significantly contribute to the hyperpolarization of tonic current. Therefore, 3 μM GABA was designated as the extrasynaptic EC_{100} (**Figure 16C**), however our previous study determined 3 μM as capable of modulating synaptic mIPSC amplitude and kinetics (section IV.1.6, **Table 3**). To delineate the synaptic/extrasynaptic receptor classes, we selected 1 μM GABA for further tonic current studies. Thus, 10 μM GABA

represents tonic current modulation whereby non-δ-containing receptors contribute to the response above the extrasynaptic GABA EC_{100} level in both WT and δKO DGGCs.

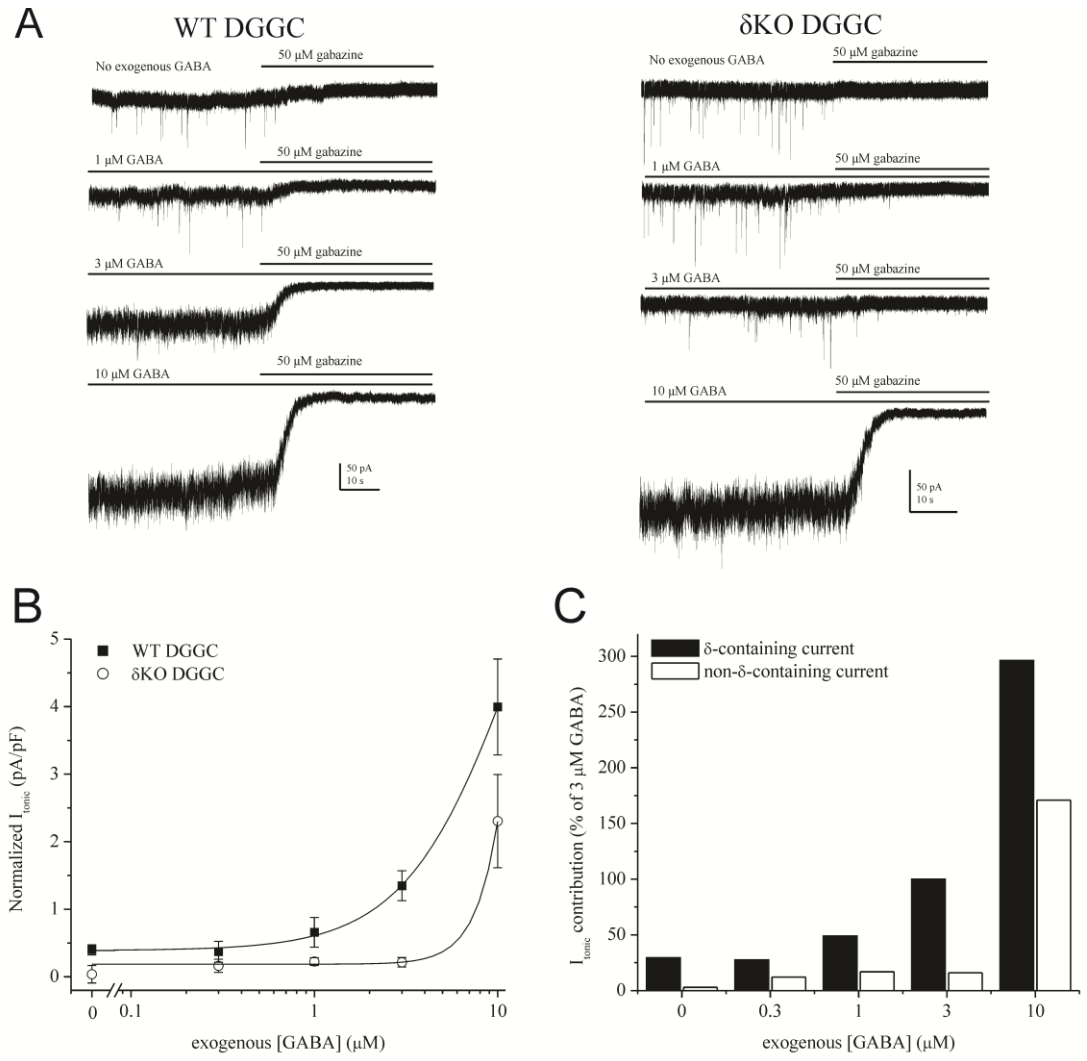


Figure 16. Concentration-related responses of GABA on δ-subunit extrasynaptic receptor-mediated tonic inhibition. ***A***, Representative whole-cell, voltage-clamp recordings of tonic current in DGGCs in response to GABA. After recording, gabazine was applied to measure total tonic shift. ***B***, GABA concentration-response curves for normalized tonic current shift (pA/pF) in WT and δKO DGGCs. ***C***, Percent tonic current contribution of δ-subunit-containing and non-δ-containing receptors relative to the 3 μM GABA response. Percent values were determined from the mean values of WT and δKO cells in B**.** GABA concentrations less than 10 μM did not elicit significant responses in δKO DGGCs, thereby denoting majority tonic current contribution by δ-containing $GABA_A$ receptors. Recordings in whole-cell mode, voltage-clamp -65mV. n = 6 – 10 cells per group and concentration.

Contribution of δ-containing receptors to $GABA_A$ receptor-mediated tonic inhibition

To confirm the relative contribution of δ-containing receptors to total I_{tonic}, we conducted whole-cell, patch-clamp experiments measuring neurosteroid-mediated tonic current in WT and δKO DGGCs in slice. Exogenous GABA (1 μM) was applied as a non-desensitizing agonist that would continuously gate extrasynaptic receptors from WT DGGCs as previously demonstrated (**Figure 16A**). After establishing a baseline holding current in the presence of GABA, neurosteroid was applied to the bath perfusion. Excessive tissue accumulation of steroid was prevented by sustaining a high rate of perfusion (2 ml/min). For each cell, rate of change was monitored in real-time and recordings were allowed to reach an asymptotic, steady-state level of neurosteroid perfusion before complete block by gabazine (50 μM) to determine total tonic current shift. Recordings were omitted in which the holding current did not reach a steady state after 3 minutes of neurosteroid perfusion. Therefore, the current responses represent positive allosteric modulation by neurosteroid. Sigmoidal curves were fitted to the concentration-response data for each drug to determine relative efficacy and potency of receptor modulation. Since a plateau response was not reached in the range of neurosteroid tested, the data could not be fit to a Hill-curve; therefore, the potency could not be determined.

From the tonic response to GABA alone, we observed that 10 μM GABA elicited strong hyperpolarizing responses representative of continuous, non-desensitizing tonic current, even in δKO DGGCs (**Figure 16B)**. Reduced tonic current and decreased neurosteroid sensitivity due to δKO is well documented (Carver et al., 2014; Mihalek et al., 1999; Stell et al., 2003; Glykys et al., 2008), however the modulatory effects on high-efficacy receptors have not been thoroughly explored. Therefore, we first assessed AP responses in WT and δKO DGGC to determine the extent of δ-subunit-specific allosteric modulation of extrasynaptic function (**Figure 17**). The average tonic response from 0.01 μM AP + 1 μM GABA application (0.86 ± 0.17 pA/pF, n = 8 cells) was greater than, but not significantly different than 1 μM GABA alone in WT DGGCs (0.66 ± 0.22 pA/pF, n = 9 cells; p = 0.4912). Increasing concentrations of AP (0.1 – 3.0 μM) elicited enhanced, potentiated responses on tonic current. The tonic current produced by allosteric AP modulation was completely diminished in δKO granule cells compared with WT (**Figure 17B**). Three μM AP + GABA significantly potentiated current within δKO DGGCs, but the tonic current response of WT neurons remained significantly greater than δKO (p = 0.0008,

n = 5-6 cells per group). The criterion for determining the δ-containing, extrasynaptic EC_{100} for allosteric modulation by AP was the highest AP concentration at which I_{tonic} in δKO DGGCs was not significantly different than condition of baseline 1 μM GABA. Based on these concentration-response data, we investigated relative percent contribution of δ-containing and non-δ-containing receptors to AP-mediated I_{tonic}, using 1 μM AP as the EC_{100} for allosteric activation (**Figure 17C**). These results suggest that neurosteroid ≤ 1 μM is selective for δ-contianing extrasynaptic receptors in modulation of tonic current in the hippocampus slice preparation.

Lack of tonic current response in dentate gyrus interneurons

To investigate the tonic inhibition in distinct cell types within the DG, we recorded I_{tonic} in a specific population of interneurons. Hippocampal interneurons have been reported to possess $GABA_A$ receptor-mediated tonic conductance, which can affect network excitability and the inhibitory drive to principal cells (Semyanov et al., 2003). Molecular layer interneurons express the α1βδ isoform extrasynaptically, but the contribution to network tonic conductance is not very well understood (Sun et al., 2004; Glykys et al., 2007). The α1βδ receptor isoform exhibits very little GABA-mediated current, but in the low-efficacy state it can be significantly potentiated by neurosteroids (Zheleznova et al., 2008; 2009). In order to better understand neurosteroid sensitivity of δ-containing extrasynaptic receptors, we compared tonic currents between WT principal granule cells and molecular layer (ML) interneurons in the slice (**Figure 18**). The cell types were visually identified by location and morphology using differential interference contrast microscopy within the slice. Whole-cell, voltage-clamp recordings were used to measure the tonic current conductances in the presence of 1 μM GABA + 3 μM AP to maximally drive channel potentiation (**Figure 18A**). DGGCs exhibited significantly greater tonic current sensitivity to AP than ML interneurons (**Figure 18B**). Baseline tonic current with GABA alone was not significantly different between DGGC and ML interneuron. The mean fold-enhancement of tonic current elicited by AP was 12.1 ± 3.1-fold increase in DGGC, whereas ML interneuron increase was only 4.3 ± 1.2-fold ($p = 0.0409$).These results are consistent with previous reports demonstrating that ML interneurons exhibit significantly less GABAergic tonic conductance than DGGCs (Glykys et al., 2008; Wei et al., 2013) and significantly lower modulation of δ-containing receptors by the α4βδ-selective modulator DS2 (Wei et al., 2013).

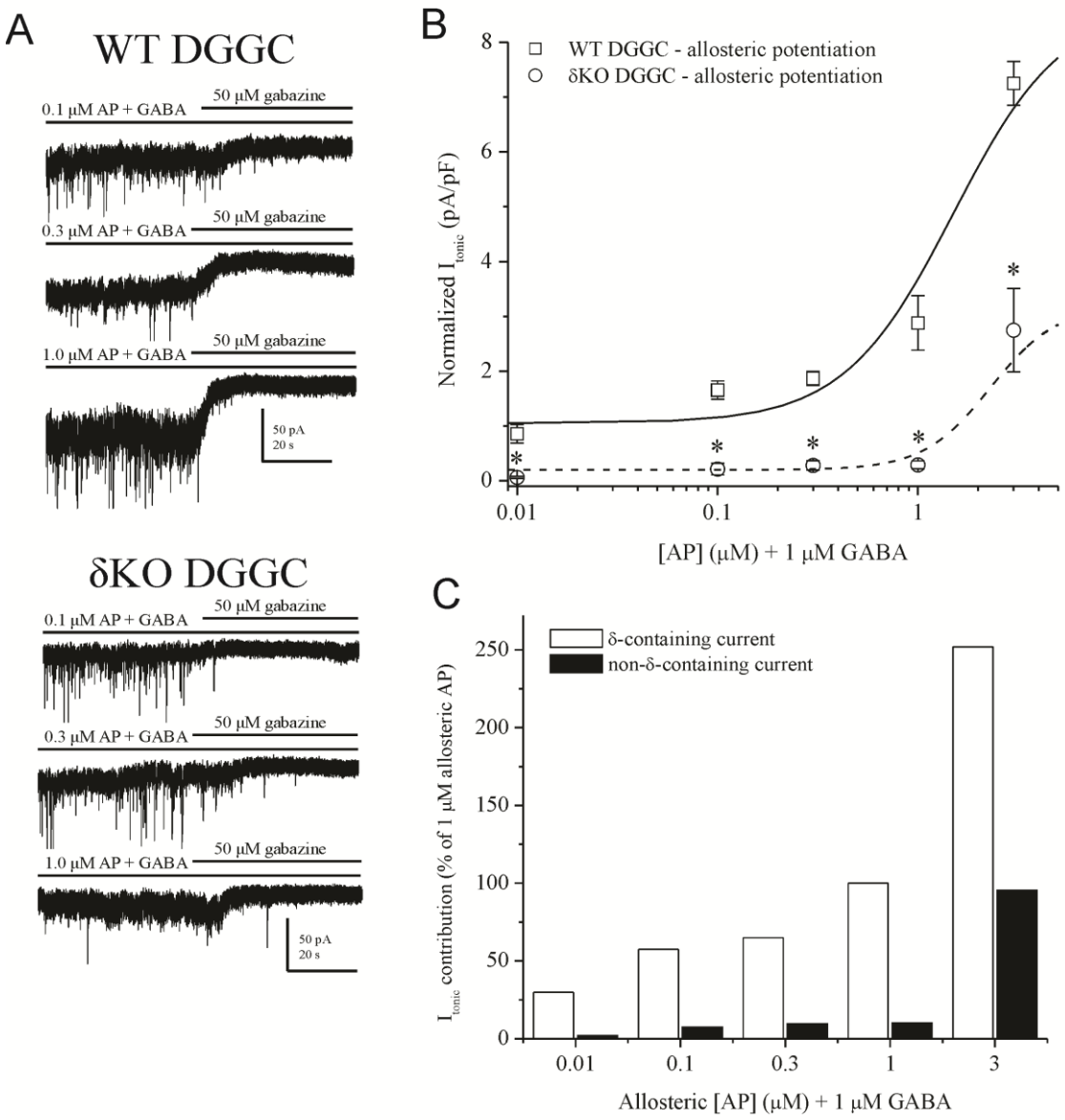


Figure 17. Contribution of δ-containing extrasynaptic receptors to total tonic inhibition elicited by AP allosteric potentiation. ***A***, Representative tonic current recordings from patch-clamped DGGCs in slice. AP was applied to the bath in addition to 1 μM GABA to measure allosteric enhancement of tonic current. 1 μM GABA, AP, and gabazine were sequentially applied in perfusion to measure tonic current shift. ***B***, AP concentration-response curves from WT (squares) and δKO (circles) DGGCs. Tonic current (pA/pF) is normalized to cell capacitance as a measure of current density. δKO DGGCs displayed highly attenuated tonic currents compared to WT. ***C***, Percent contribution of allosteric AP tonic current from δ-containing and non-δ-containing receptors, relative to 1 μM allosteric AP, which was determined to be the constrained maximum allosterically-active concentration. DGGCs from brain slice of diestrus female mice, recorded in whole-cell mode, voltage-clamped at -65mV. n = 6-8 cells per concentration and group.

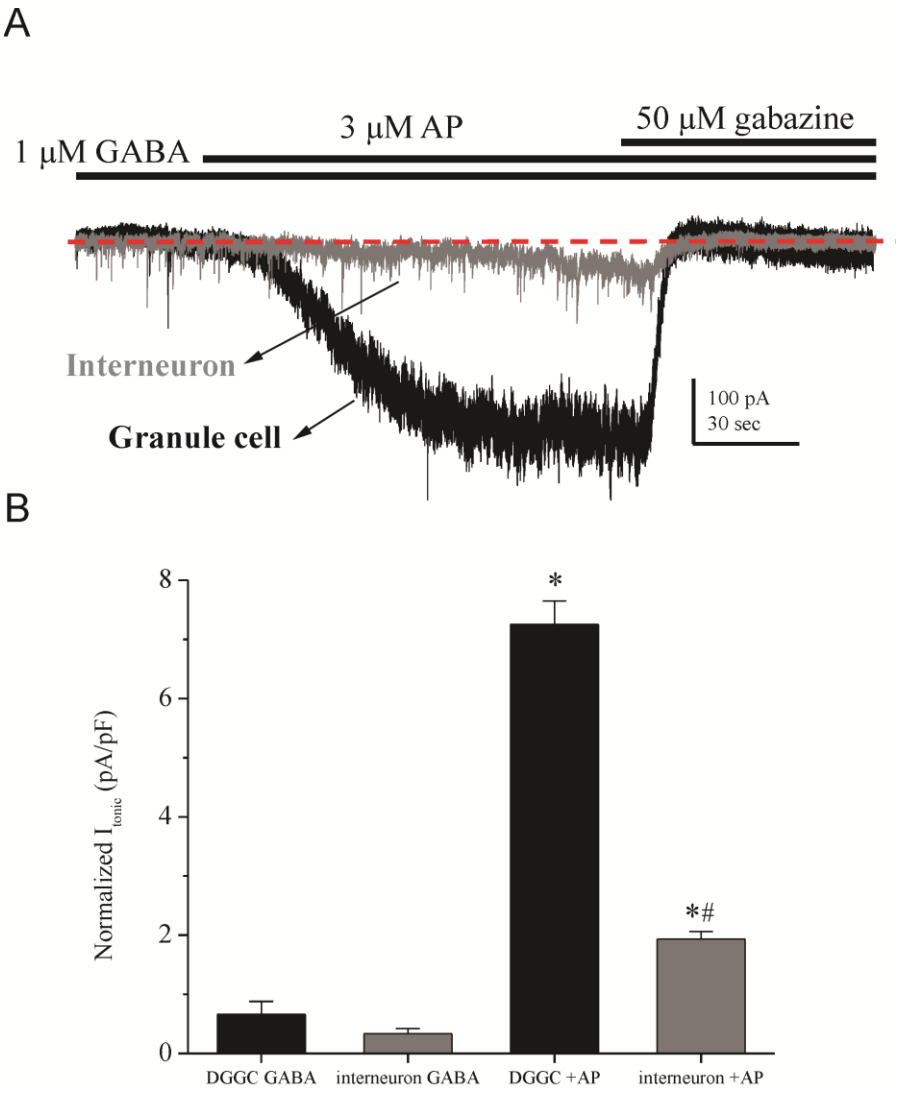


Figure 18. ML interneurons exhibit less tonic current modulation elicited by AP compared with DGGCs. ***A***, Representative traces for 1 μM GABA + 3 μM AP application in slice recording of extrasynaptic, tonic current. Traces from dentate gyrus granule cell (black trace) and molecular layer interneuron (gray trace) are superimposed to show contrast, with equal scale. The red dashed line describes the holding current level after gabazine block of GABAergic current. The DGGC exhibited a robust response to 3 μM AP (Δ279 pA), whereas the ML interneuron cell had reduced tonic current response (Δ41 pA). ***B***, Quantification of tonic current due to 1 μM GABA or 3 μM AP allosteric modulation of 1 μM GABA. Interneurons exhibited signficantly less normalized tonic current than granule cells (* $p < 0.01$ vs. GABA control, # $p < 0.01$ vs. DGGC + AP). Mean tonic shift (pA) was also significantly different for AP potentiation (DGGC: 223.7 ± 26.6 pA, ML interneuron: 28.3 ± 1.8 pA; $p < 0.01$). Cells were recorded from adult WT female (diestrous stage) hippocampus slices, voltage-clamped (-65mV) in whole-cell mode. n = 5-6 cells per group.

Lack of δ-containing receptor-mediated tonic current response in CA1 pyramidal cells

To ascertain whether CA1 pyramidal neurons exhibit any tonic conductances mediated by δ-containing receptors, we recorded I_{tonic} in CA1 cells in comparison with DGGCs. The α5βγ2 receptor isoform contributes a majority of the extrasynaptic, tonic current in CA1 pyramidal cells (Glykys et al., 2008). The δ-containing receptors are neither highly expressed (Peng et al., 2002), nor functionally significant to tonic current modulation in CA1 (Glykys et al., 2008; Stell et al., 2003; Carver and Reddy, 2013). In order to expound on the contribution of δ-containing receptors to regional specificity of extrasynaptic inhibitory function, we compared THIP modulation of tonic currents in CA1 pyramidal cells and DGGCs. Whole-cell, voltage-clamp recordings were obtained to measure THIP-dependent tonic current shift in the slice (**Figure 19**). We compared concentration-responses between 0.03 and 1 μM THIP to isolate a concentration range of the compound that is δ-selective (Meera et al., 2011). Across all concentrations, CA1 pyramidal cells had a reduced tonic current response compared with DGGCs. At 0.3 and 1 μM THIP, the normalized tonic current was significantly different between CA1 and DGGC. Overall, these results confirm emerging evidence that δ-containing extrasynaptic receptors provide a unique inhibitory role that is region- and cell-specific within the hippocampus.

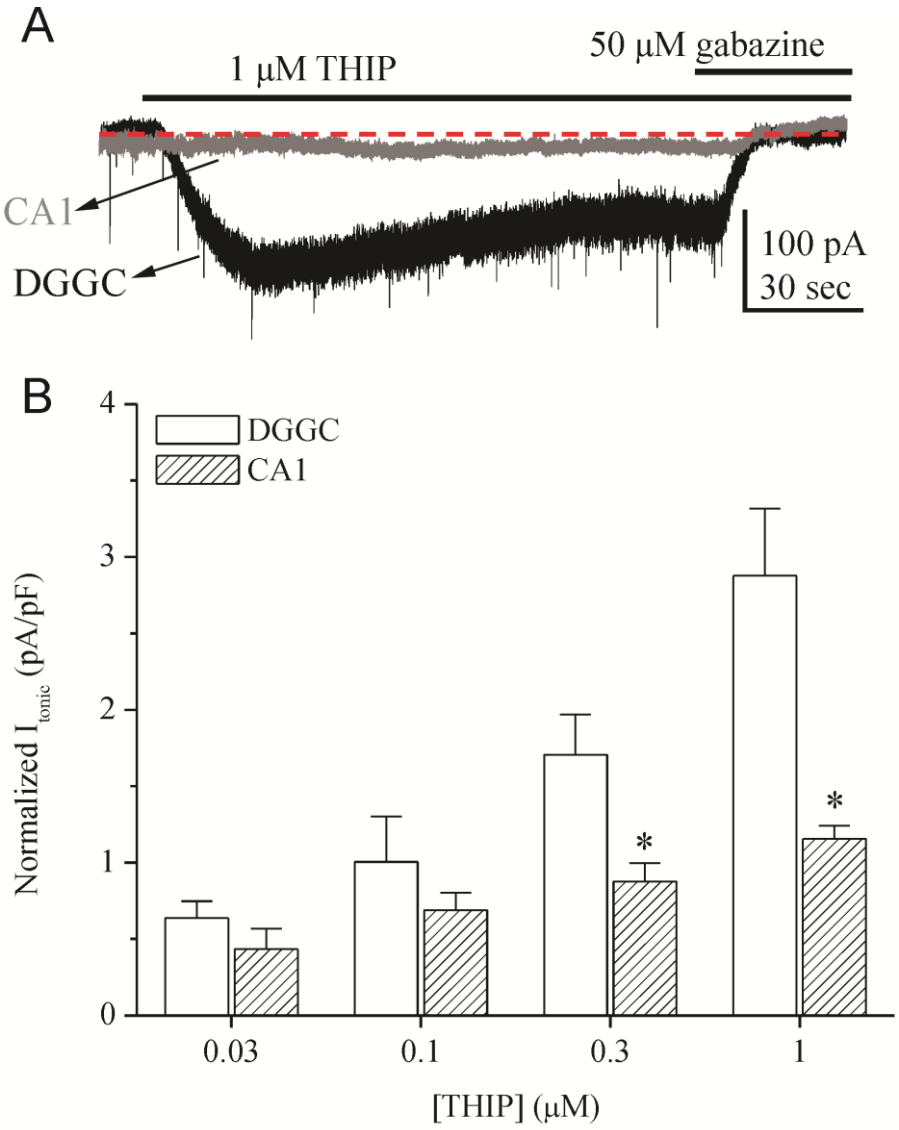


Figure 19. Lack of tonic inhibition mediated by δ-containing extrasynaptic receptors in CA1 neurons elicited by δ-selective THIP. ***A***, Representative traces of current recordings within slice of CA1 pyramidal cell (*gray trace*) or DGGC (*black trace*) upon modulation of tonic inhibition by 1 μM THIP. The DGGC exhibits a robust increase to tonic current elicited by THIP, whereas the CA1 cell has very little response. ***B***, Concentration-response to THIP in DGGC and CA1 principle cells as quantified by normalized tonic current (pA/pF). THIP responses were greater in DGGC cells at all concentrations, however, 0.3 – 1 μM THIP significantly enhanced extrasynaptic tonic currents in DGGCs above that of CA1 pyramidal cells. Cells were recorded from WT adult female (diestrous stage) hippocampus slices, voltage-clamped -65mV in whole-cell mode. * $p < 0.05$ vs. DGGC, n = 4-7 cells per concentration and cell-type.

Direct responses of neurosteroid on tonic inhibition

We examined tonic currents of granule cells in which $GABA_A$ receptors are directly activated by neurosteroid without GABA gating. The concentration of neurosteroid able to directly gate $GABA_A$ receptors ($\geq$ 1 μM) is greater than the observed physiological range of endogenous neurosteroids (0.1 – 0.3 μM) (Carver and Reddy, 2013; Majewska et al., 1986). However, exogenous or therapeutic administration of neurosteroids can reach sufficiently high concentrations within the brain to achieve direct activation of $GABA_A$ receptors, anticonvulsant activity, sedation, and motor impairment (Reddy, 2003ab). We explored a range of applied AP without exogenous GABA to assess the direct modulation of tonic current by this neurosteroid (**Figure 20**). AP alone significantly enhanced tonic current above the baseline control level. The concentration-response of directly-activating current was reduced compared to the allosteric curve. Next, we investigated the relative percent contribution of δ-containing and non-δ-containing receptors to AP-mediated tonic current. We determined 3 μM AP to be the maximum concentration for direct activation of current. We demonstrate that at sub-micromolar concentrations, AP can enhance and potentiate tonic current in a GABA-deficient environment. However, accumulation of neurosteroid within the neuronal membrane likely contributes to this direct-binding activity, as enhancement of tonic current proceeds gradually (Akk et al., 2005).

In δKO DGGCs, a small fraction of residual, extrasynaptic current remains, due to extrasynaptic α_5-containing receptors (Glykys et al., 2008). However, the neurosteroid structure-activity for extrasynaptic α_5- and γ-containing receptors is much lower than those incorporating δ (Brown et al., 2002; Stell et al., 2003). The residual tonic current response was measured in δKO DGGCs during application of AP without exogenous GABA. Direct tonic current recordings displayed striking attenuation of current response in δKO compared to WT DGGCs (**Figure 20**). In prolonged application of neurosteroid to allow membrane accumulation, we did not observe any gradual enhancement of extrasynaptic current by 1 μM AP in δKO cells. At 3 μM AP, δKO tonic current was significantly increased, however, non-δ-containing receptors only contributed to 29.0 % of the tonic current achieved by δ-containing receptors from WT. Taken together, these results suggests an obligatory role of δ-subunit transduction for the neurosteroid direct activation of tonic currents.

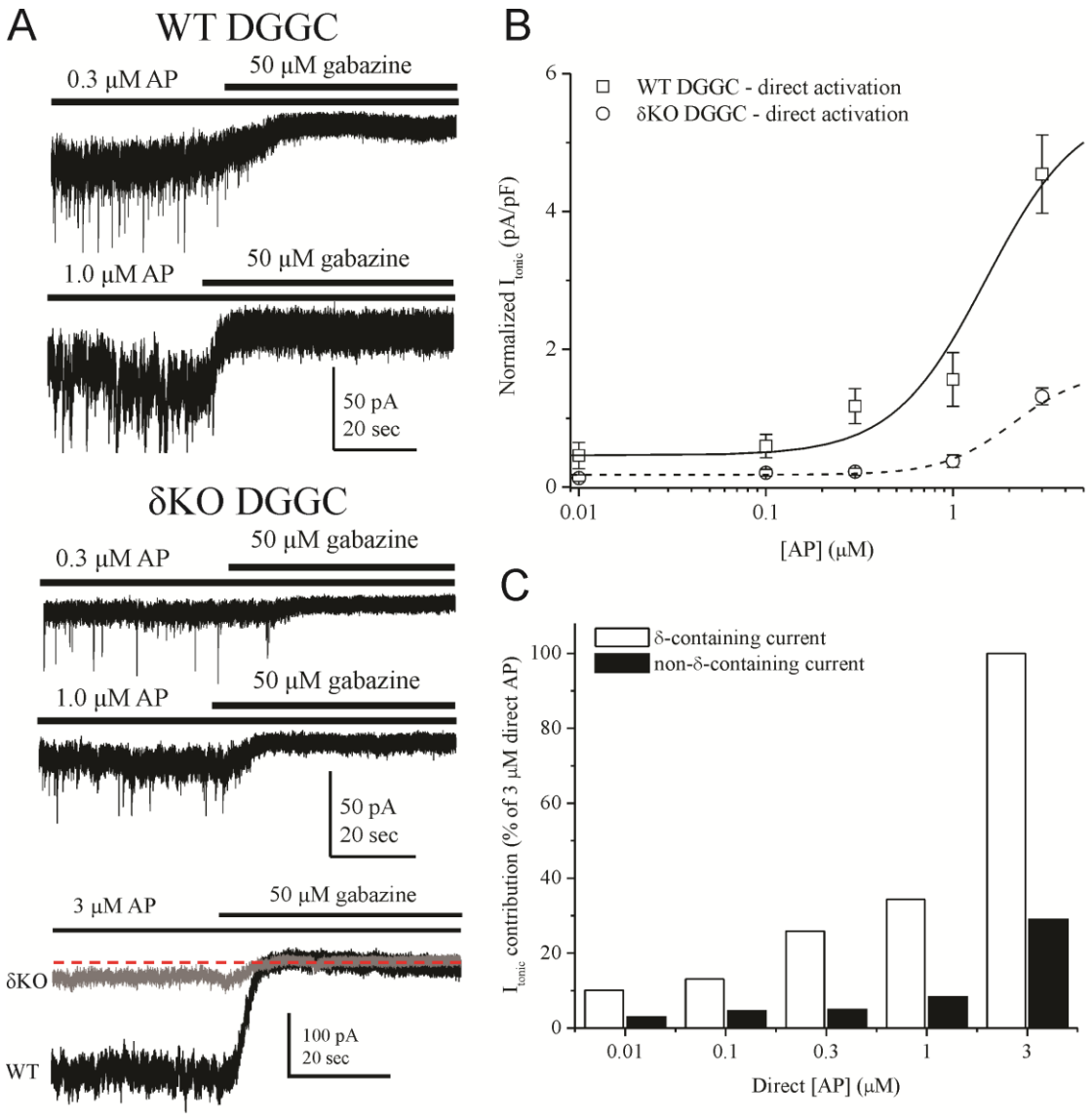
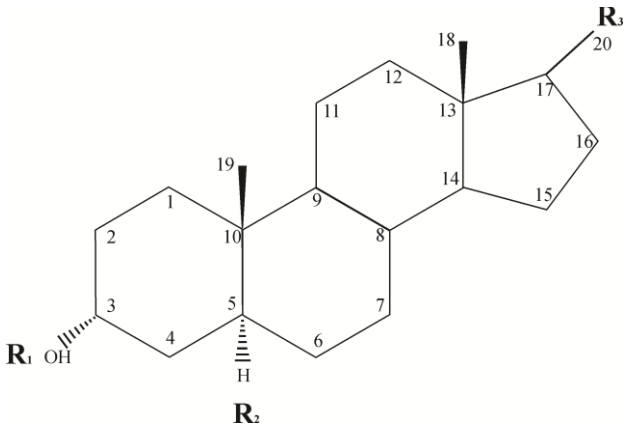


Figure 20. Contribution of δ-containing extrasynaptic receptors to total tonic inhibition elicited by AP direct activation. ***A***, Representative tonic current recordings from patch-clamped WT and δKO DGGCs in slice with AP (0.3 or 1.0 μM). AP alone (no GABA) followed by gabazine were applied to measure tonic current shift of extrasynaptic receptors directly-activated by AP. The bottom superimposed trace displays same-scale, 3 μM AP-dependent tonic currents from a WT DGGC (black trace) and a δKO DGGC (gray trace). The red dashed line indicates the holding current level upon gabazine block of GABAergic current. In addition, the increased RMS noise of channel conductance is apparent in the WT recordings, due to δ-containing receptor activation. ***B***, Direct AP concentration-response curves from WT (squares) and δKO (circles) DGGCs. Direct activation denotes that the neurosteroid was applied to bath solution alone, without any exogenous GABA to measure directly-activating extrasynaptic currents. ***C***, Relative percent contribution of δ-subunit and non-δ-subunit receptors to directly-activating tonic current by AP, relative to WT 3 μM AP alone.

Table 4. Chemical structures of neurosteroids tested for extrasynaptic activity



Steroid	C3 position (R_1)	C5 position (R_2)	C17 position (R_3)	Key Structural Significance
Allopregnanolone (AP)	α-OH	α-H	-$COCH_3$	3α-hydroxyl, 5α-reduction,17-acetyl
Ganaxolone (GNX)	α-OH, β-CH_3	α-H	-$COCH_3$	3β-methyl
5β-allopregnanolone (5β-AP)	α-OH	β-H	-$COCH_3$	5β-reduction
3β-allopregnanolone (3β-AP)	β-OH	α-H	-$COCH_3$	3β-hydroxyl
Tetrahydrodeoxycorticosterone (THDOC)	α-OH	α-H	-$COCH_2OH$	21-hydroxyl
Alfaxolone (ALFX)	α-OH	α-H	-$COCH_3$	11-carbonyl
Androstanediol (AD)	α-OH	α-H	β-OH	17-hydroxyl
Androsterone (AN)	α-OH	α-H	=O	17-carbonyl
Etiocholanolone (ETIO)	α-OH	β-H	=O	17-carbonyl, 5β-reduction
ORG-20599	α-H	α-H	-$COCH_2Cl$	2β-(4-morpholinyl), 17-acyl chloride

Comparative allosteric responses of neurosteroids on tonic inhibition

To identify the key neurosteroid structures contributing to $GABA_A$ receptor tonic inhibition, we compiled a library of neurosteroids with distinct structural features (**Table 4**). Each neurosteroid was evaluated for its ability to potentiate tonic current. Concentration-dependent, normalized tonic current responses are shown in **Figures 21-23**.

The concentration-response of 3α5α-AP displayed significantly greater functional efficacy than other endogenous neurosteroids tested (**Figure 21**). THDOC, derived from deoxycortcosterone, modulated significantly less tonic current in the 0.1-0.3 μM range, compared with AP. However, at 1.0-3.0 μM, THDOC and AP potentiated similar levels of tonic current. 5β-AP, which differs from AP by a C5-position β-hydrogen, had a reduced tonic current response at all concentrations tested. The 3β-AP steroid, which was previously observed to have reduced efficacy on whole-cell or synaptic currents (Gee et al., 1988; Kokate et al., 1994), was not functionally active on tonic currents within the slice, even at 10 μM when applied with GABA (0.51 ± 0.14 pA/pF, mean tonic shift: 15.7 ± 4.3 pA).

Next, synthetic neurosteroids were compared in their ability to enhance extrasynaptic tonic current (**Figure 22**). GNX differs from AP by the addition of a C3-position β-methyl group. The ORG-20599 compound includes a C2-position β-4-morpholinyl group as well as modification to the 17β group by acyl chloride. In comparison with synthetic neurosteroid structures, AP had similar response profiles to GNX and ORG-20599. However, ALFX, which includes a C11-position ketone, displayed reduced tonic currents and an overall decrease in δ-mediated extrasynaptic receptor efficacy.

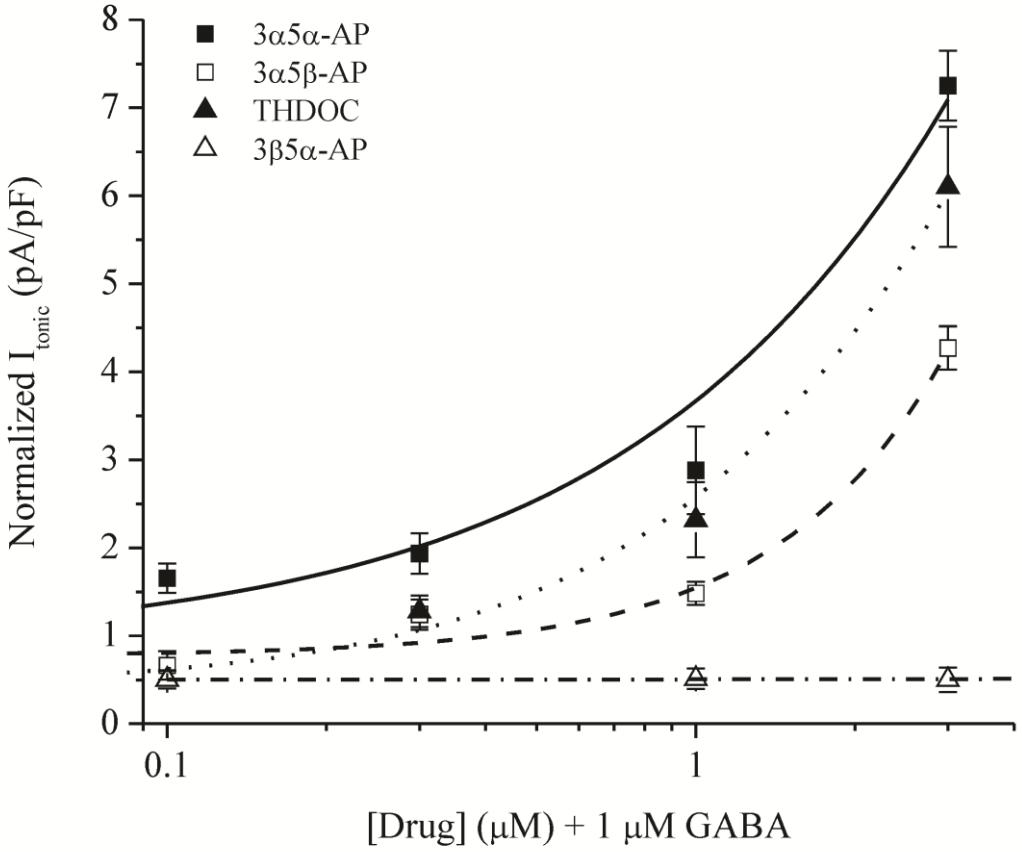


Figure 21. Pregnane neurosteroid structure-activity relationships on δ-subunit extrasynaptic receptor-mediated tonic inhibition. Concentration-response curves of endogenous pregnane steroids as measured by tonic current from DGGCs (pA/pF) from patch-clamp recordings. Lines represent non-linear curve fit by the neurosteroids AP (*solid*), 5β-AP (*dash*), THDOC (*dot*), or 3β-AP (*dash-dot*). Application of 3 μM neurosteroid is signified by an exponential increase to tonic current. X-axis: + 1 μM GABA denotes allosteric modulation by co-application of the drug compound with GABA in the bath perfusion. All data derived from female WT DGGCs. All cells voltage-clamped in whole-cell mode, at -65mV; n = 5-10 cells per concentration and drug.

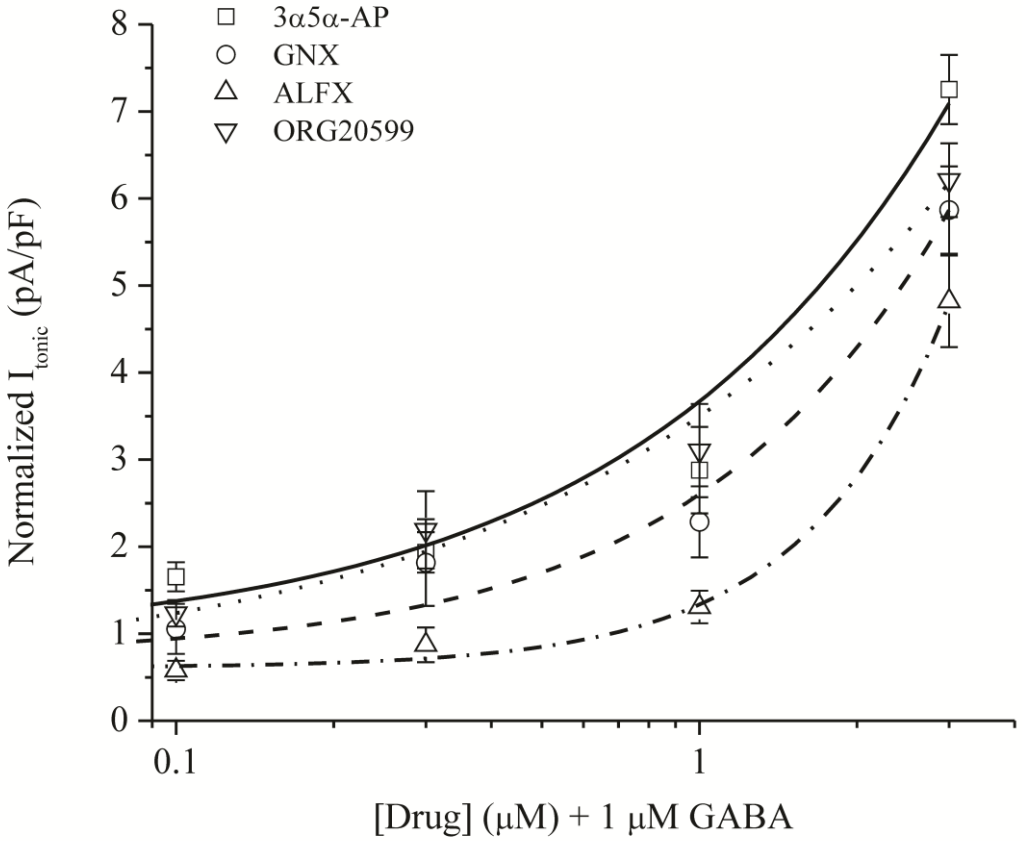


Figure 22. Synthetic neurosteroid structure-activity relationships on δ-subunit extrasynaptic receptor-mediated tonic inhibition. Concentration-response curves of synthetic pregnane steroids as measured by tonic current from DGGCs (pA/pF) from patch-clamp recordings. AP responses are included in the figure as a comparative, prototypical neurosteroid. Lines represent non-linear curve fit by the neurosteroids AP (*solid*), GNX (*dash*), ALFX (*dash-dot*), or ORG-20599 (*dot*). X-axis: + 1 μM GABA denotes allosteric modulation by co-application of the drug compound with GABA in the bath perfusion. All data derived from female WT DGGCs. All cells voltage-clamped in whole-cell mode, at -65mV; n = 5-10 cells per concentration and drug.

The androstane neurosteroids display weakly positive allosteric modulation of $GABA_A$ receptors (Reddy and Jian, 2010), but have not been explored with regard to extrasynaptic structure-activity. The 17β-ketosteroids androsterone (AN) and etiocholanolone (ETIO) are the main metabolites of testosterone. Androstanediol (AD) is derived from dihydrotestosterone, the 5α-reduced metabolite of testosterone. We evaluated tonic currents from DGGCs in response to AD, AN, and ETIO (**Figure 23**). Compared with AP (1 μM), the average tonic currents of AD, AN, and ETIO were smaller by a factor of 3.1, 4.5, and 4.9, respectively. Overall, these steroids have lesser potency on δ-containing extrasynaptic receptors than the pregnane neurosteroids, suggesting weaker binding affinity and/or transduction.

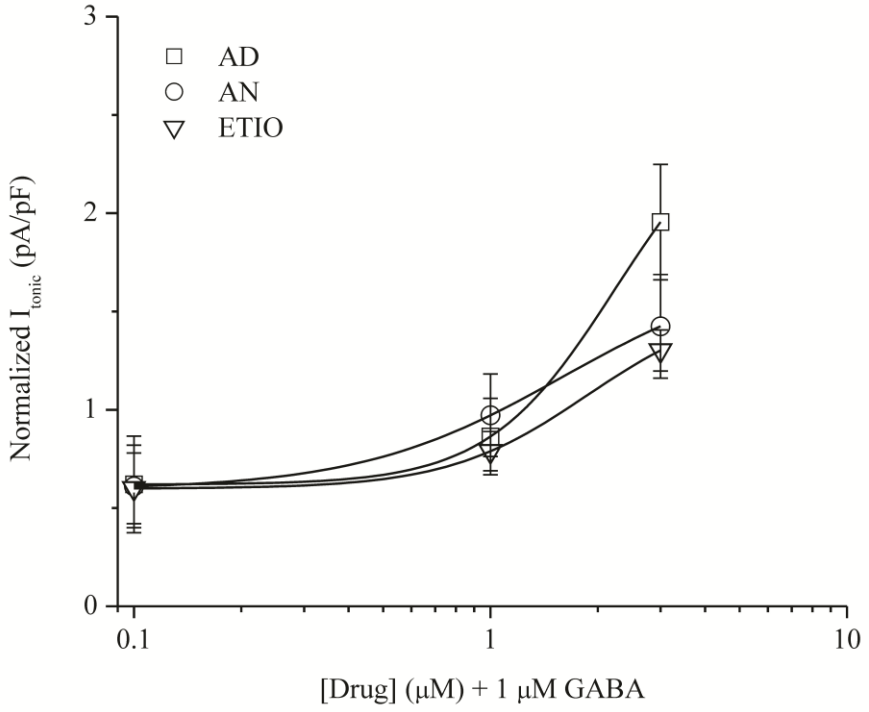


Figure 23. Androstane neurosteroid structure-activity relationships on δ-subunit extrasynaptic receptor-mediated tonic inhibition. Concentration-response curves of endogenous androstane steroids as measured by tonic current from DGGCs (pA/pF) from patch-clamp recordings. The androstane neurosteroids have a right-shifted potency and extrasynaptic receptor affinity. + 1 μM GABA denotes allosteric modulation by co-application of the drug compound with GABA in the bath perfusion. All data derived from female (diestrous stage) WT DGGCs. All cells voltage-clamped in whole-cell mode, at -65mV; n = 4-8 cells per concentration and drug. AD = androstanediol (*squares*). AN = androsterone (*circles*). ETIO = etiocholanolone (*triangles*).

To compare the extrasynaptic, tonic current contribution of each structurally distinct neurosteroid, we analyzed the differences in fold-potentiation of the GABA (1 μM) response (**Table 5**). At the 0.1 μM concentration, AP (2.5-fold) displayed significantly greater potentiation, as GNX (1.6-fold) and ORG-20599 (1.9-fold) showed moderate potentiation of extrasynaptic GABA. At 0.3-3 μM concentrations, GNX and ORG-20599 exhibited comparable potentiation of tonic current to the responses by AP. Interestingly, 0.1 μM THDOC did not elicit potentation of tonic current, indicating that the C21-hydroxylation modification confers lower extrasynaptic receptor efficacy or potency. This was confirmed by the diminished response of THDOC at higher concentrations, compared to the pregnane neurosteroids with C17 structural features similar to AP (see Table 4). At 0.3 μM and above, all pregnane neurosteroids tested except for 3β-AP exhibited significant potentiation of the extrasynaptic GABA response. Androstane neurosteroids did not significantly enhance GABAergic tonic current at concentrations under 1 μM, and only modestly potentiated current at 3 μM.

Interestingly, 3 μM pregnane neurosteroids displayed exponential potentiation of tonic currents. This suggests that higher concentrations directly gate low-occupancy receptors, producing a biphasic effect of receptor activation. Therefore, we designated 1 μM pregnane neurosteroid to be the constrained maximum response for extrasynaptic receptors in allosteric activity. This was consistent in our extensive concentration-response study of both allosteric potentation and direct-activation by AP in determination of δ-containing receptor contribution to tonic current (**Figure 17,18**). However, relative efficacy of neurosteroid to potentiate functional inhibition was determined for each structure based on normalized data for the entire range of concentrations tested (0.1 - 3 μM). Higher concentrations exceed the physiological range of relevance of this study beyond that of feasible limits (Gee et al., 1988; Reddy and Rogawski, 2012), but pharmacological doses of neurosteroids remain salient to understanding their therapeutic application in the brain.

Table 5. Neurosteroid-mediated potentiation of extrasynaptic, tonic currents by 1 μM GABA.

Compound	0.1 μM neurosteroid potentiation	0.3 μM neurosteroid potentiation	1.0 μM neurosteroid potentiation	3.0 μM neurosteroid potentiation
AP	2.50	2.94	4.36	10.98
5β-AP	0.76	1.88	2.24	6.47
3β-AP	0.76	0.76	0.76	0.77
THDOC	1.01	1.94	3.52	9.24
GNX	1.61	2.76	3.47	8.89
ALFX	0.88	1.32	1.98	7.30
ORG-20599	1.88	3.32	4.70	9.41
AD	0.94	1.09	1.30	2.95
AN	0.92	n.d.	1.47	2.15
ETIO	0.91	n.d.	1.20	1.97

Values derived from fold-potentiation of mean, normalized tonic current response to 1 μM GABA, (I_{NS}/I_{GABA}). I_{NS} is the mean, normalized tonic current response to neurosteroid at the given concentration + 1 μM GABA from Figures 21-23. Tonic current responses were recorded from voltage-clamped(-65mV) DGGCs from WT, female (diestrous stage) mice. All mean values are representative of 4-8 cells per neurosteroid and concentration.

Tonic current responses of non-steroidal, δ-selective compounds DS2 and THIP

We investigated DS2 (4-chloro-*N*-[2-(2-thienyl)imidazo[1,2-a]pyridin-3-yl]benzamide) as a comparative δ-selective agent and non-steroidal, allosteric modulator (Wafford et al., 2009; Jensen et al., 2013). DS2 selectivity was previously determined in recombinant cells expressing $\alpha_4\beta\delta$ receptors and thalamic relay neurons, however it has not been analyzed in tonic currents from native DGGCs in the slice. In extrasynaptic function as an allosteric modulator, DS2 was less potent in modulation and affinity than the pregnane class of neurosteroids, represented by the prototypical AP (**Figure 24)**. In δKO DGGCs, DS2 did not significantly potentiate tonic current greater than the 1 μM GABA baseline responses (10 μM DS2 + 1 μM GABA: 7.3 ± 4.3 pA, 1 μM GABA: 6.9 ± 2.1 pA; n = 6-7 cells per group, p = 0.9317). These responses confirm the δ-selectivity of DS2 in native DGGC in the slice. Previous studies established conservation of residues on the α_4 subunit necessary for neurosteroid binding (Hosie et al., 2006; Jensen et al., 2013). In mutation of these residues, Jensen and colleagues show that the GABA-enhancing action of DS2 did not rely on these residues, whereas the mutation of the α_4 subunit resulted in AP insensitivity. Thus, the decreased potency of DS2 compared to neurosteroids suggests a lower affinity for extrasynaptic $GABA_A$ receptors and a different binding site from neurosteroids.

THIP is a selective low-efficacy modulator of δ-containing receptors at sub-micromolar concentrations (Mortensen et al., 2010). THIP (no GABA in bath perfusion) was investigated for tonic current modulation and compared in its potency and efficacy with AP and DS2 (**Figure 24**). The concentration-response of THIP in the 0.1 – 1 μM range was less efficacious than AP, but more potent than DS2 allosteric modulation. However, 1 μM THIP tonic current in δKO DGGCs (0.32 ± 0.08 pA/pF) was significantly lower compared to WT DGGCs (2.57 ± 0.48 pA/pF, n = 6-7 cells per group, p = 0.0013). Increased concentration of THIP (10 μM) in δKO (2.68 ± 0.41 pA/pF) produced tonic currents comparable to 1 μM THIP in WT cells. These results indicate a single order of magnitude, rightward shift of THIP affinity for δ-deficient DGGCs.

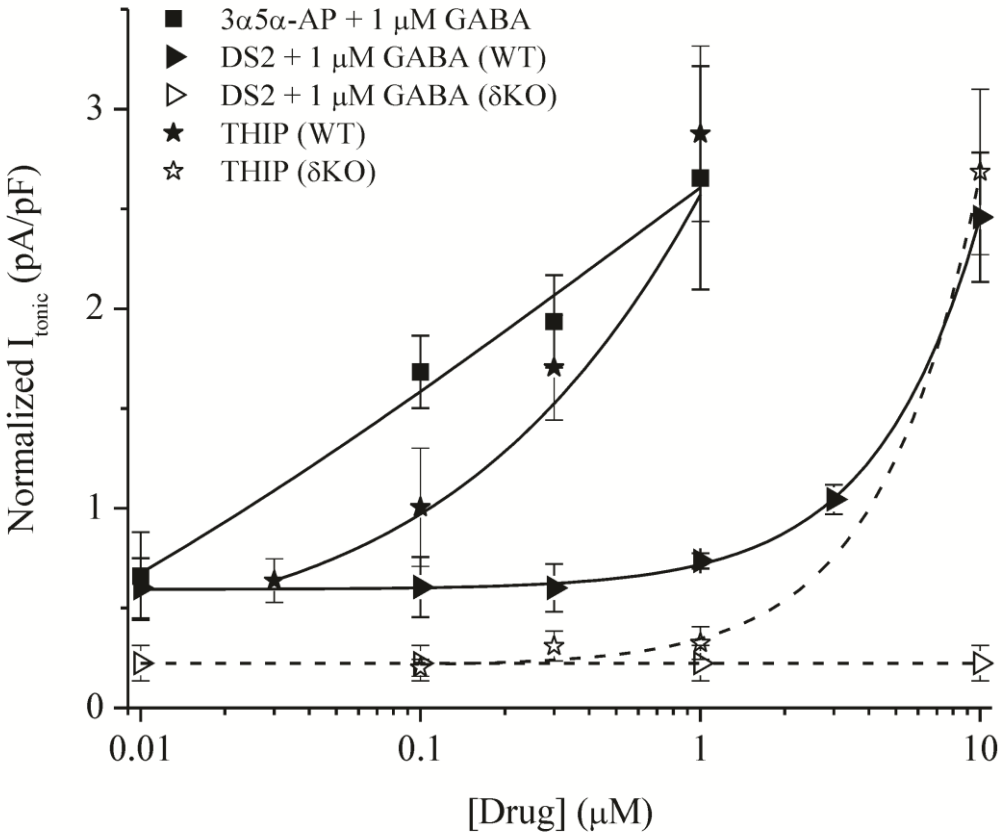


Figure 24. Comparative efficacies of δ-subunit selective compounds on tonic inhibition mediated in DGGCs. Tonic current responses for the δ-selective compounds THIP (*stars*) via direct activation and DS2 (*triangles*) allosteric modulation in comparison with AP (*squares*). Both WT (*closed symbols*) and δKO (*open symbols*) DGGC responses are depicted. The AP concentration-response is from WT DGGCs. Legend: + 1 μM GABA denotes allosteric modulation by co-application of the drug compound with GABA in the bath perfusion, whereas THIP was applied alone in bath solution. All data derived from female DGGCs. All cells voltage-clamped in whole-cell mode, at -65mV; n = 6-10 cells per concentration and drug.

IV.2.4 Neurosteroid structure-activity relationship in the 6-Hz seizure model

To study the *in vivo* anticonvulsant effects of neurosteroids for discerning structure-activity relationship, we used the 6-Hz model of partial seizures (Barton et al., 2001). Previous studies have used 6-Hz stimulation to characterize the anticonvulsant profiles of neurosteroids within mice (Kaminski et al., 2004). To test the behavioral sensitivity to 6-Hz stimulation, we first determined incidence of seizures as a function of current intensity in mice (**Figure 25A**). The CC_{50} value (current intensity at which 50% of the animals experienced seizure) was determined to be 16.5 mA for WT male mice and 10.4 mA for WT female mice (**Figure 25B**). Current intensity of 32 mA elicited seizures in 100% of the population of WT male and female control mice injected with vehicle, and further drug studies were conducted at this level of current. Interestingly, within δKO mice, we were unable to achieve a 100% seizure outcome in the animals, even in current intensities up to 100 mA in the 6-Hz model. Genetic background and its alteration have influence of seizure threshold (Leclercq and Kaminski, 2015). This suprising result precluded the further use of knockouts in the 6-Hz model, as the current intensity eliciting seizures in 100% of the population was necessary to gauge anticonvulsant effect of the neurosteroids. Therefore, seizure testing was carried out by other experimental means of convulsance for germline δKO mice in comparison with WT counterparts in later studies.

WT mice were treated with neurosteroid 15 minutes prior to 6-Hz electrical stimulation. After each stimulation, incidence of seizure protection was recorded as an all-or-nothing event (see Methods section III.5 for scoring criteria). Dose-response curves were derived for each neurosteroid drug (**Figure 26**), and potency was determined as ED_{50} (effective dose), which represents the dose that is protective for 50% of the animals (**Table 6**). In WT female mice, GNX had the greatest potency in protecting from seizures. AP and its 5β epimer had similar potency in seizure control. THDOC potency was slightly right-shifted, wheras both AFX and ORG-20599 performed worse in control of seizures. Overall, these profiles are similar to previous reports of seizure modulation in the 6-Hz model in terms of relative structure-activity (Kaminski et al., 2004). Our experiments differ in that neurosteroid was administered subcutaneously, thereby providing a higher dose available to the brain. Based on our observations, the potency achieved through neurosteroid seizure protection is greater than previously attained in other reports due to experimental constraints.

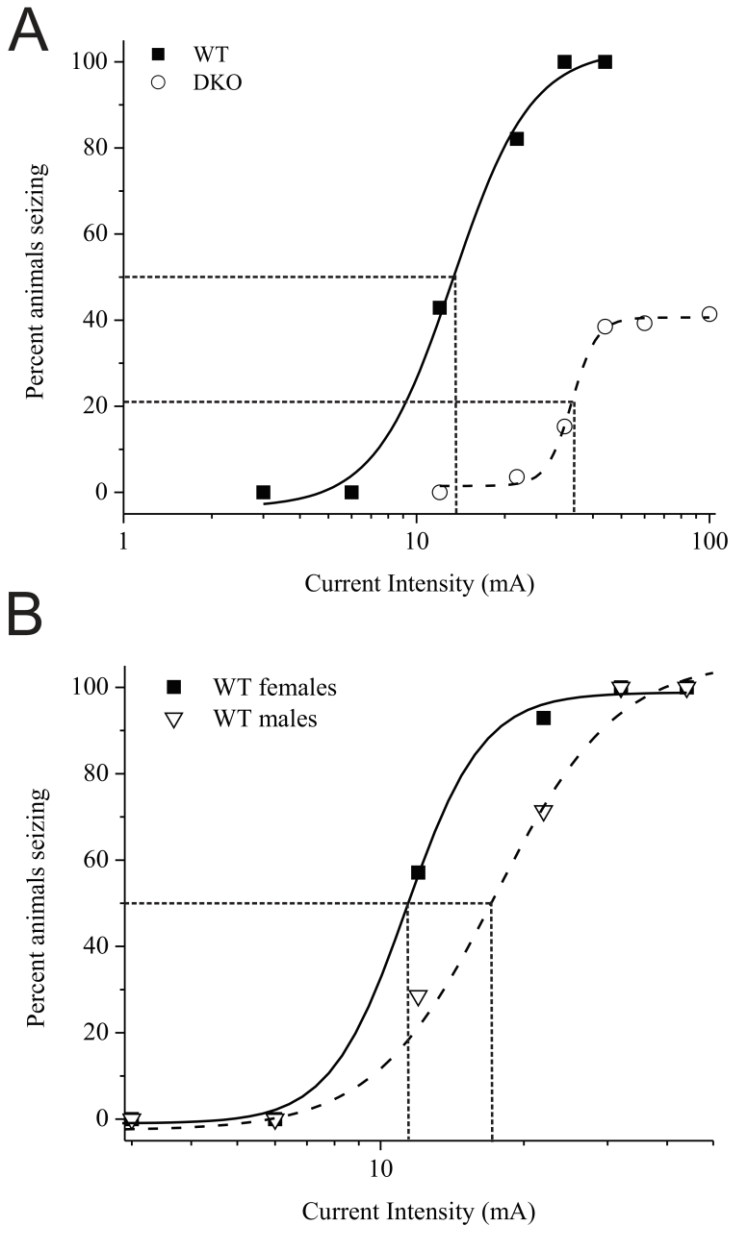


Figure 25. Behavioral sensitivity of mice in the 6-Hz seizure model. ***A***, Percent animals responding to 6-Hz stimulation as a function current intensity delivered through shock. 100% of WT animals had reliable seizure activity at 32 mA, whereas δKO mice did not respond to stimulations, even at 100 mA current. Therefore, EC_{50} could not be derived for knockout animals. Dashed line perpendicular to the fitted data represents the current intensity at which 50% of animals experienced seizures. ***B,*** Stratified male and female WT mice response to 6-Hz current-intensity. WT female mice were significantly more sensitive to stimulation, however 100% seizure response was reached at 32 mA current intensity. n = 10 animals per group.

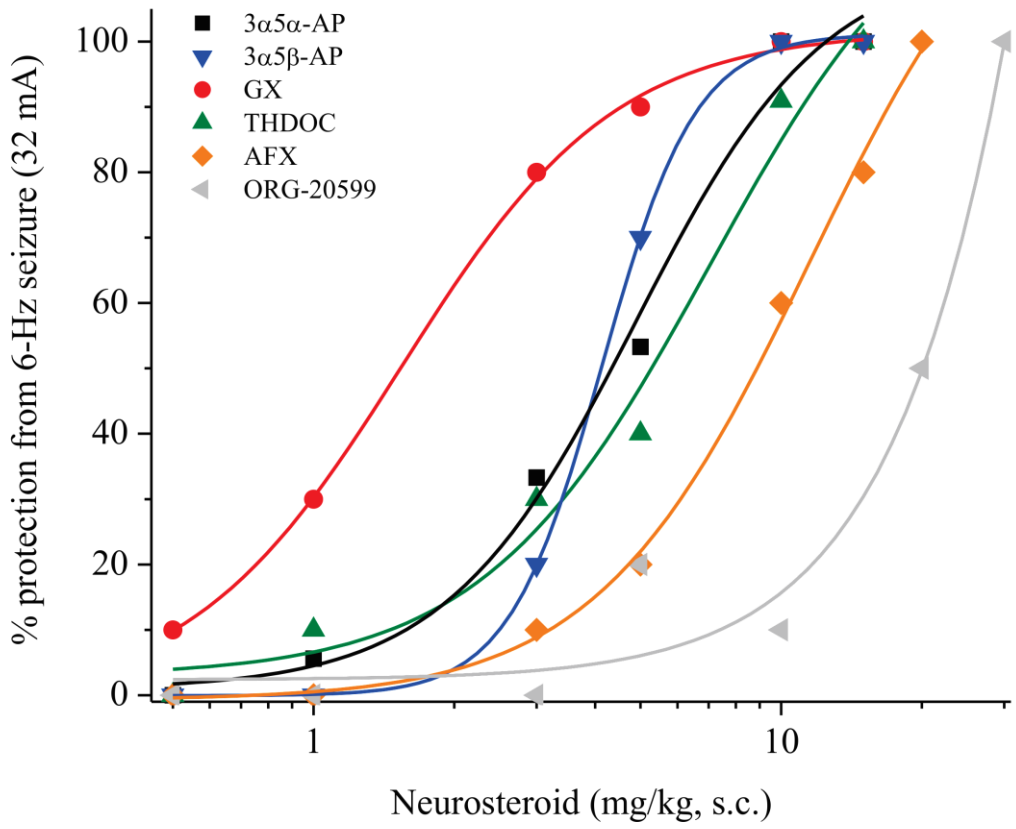


Figure 26. Neurosteroid structure-activity curves in the 6-Hz seizure model. Dose-response curves for neurosteroid modultation of 32 mA, 6-Hz induced seizures in WT female mice. Neurosteroids were applied (s.c.) 15 min. prior to stimulation. Seven structurally distinct pregnane-class neurosteroids were compared in seizure protection, using potency (ED_{50}, see **Table 6**) as the main index of anticonvulsance *in vivo* (3β-AP not shown as it did not provide full protection, even at 100 mg/kg dose). n = 10 animals per group.

Table 6. Correlation between neurosteroid modulation of tonic current and anticonvulsant profiles in the 6-Hz seizure model in mice.

Compound	$E_{1\ \mu M}$ (pA/pF)	$E_{1\ \mu M}$ (pA)	$E_{3\ \mu M}$ (pA/pF)	$E_{3\ \mu M}$ (pA)	$EF_{(2\text{-fold GABA})}$ (nM)	$EF_{(3\text{-fold GABA})}$ (nM)	6-Hz test ED_{50} (mg/kg)
AP	2.88 ± 0.50	100.6	7.25 ± 0.40	223.7	80	290	4.2 (2.7-5.8)
GNX	2.29 ± 0.41	64.0	5.87 ± 0.50	176.1	290	650	1.5 (1.3-1.7)
5β-AP	1.48 ± 0.13	44.4	4.27 ± 0.25	181.7	780	1380	7.7 (6.6-8.8)
3β-AP	0.50 ± 0.12	15.3	0.51 ± 0.14	15.7	> 10000	> 10000	> 100
THDOC	2.32 ± 0.43	66.6	6.10 ± 0.68	183.0	410	760	5.0 (2.6-7.4)
ALFX	1.31 ± 0.19	40.9	4.82 ± 0.53	144.6	990	1500	8.8 (6.1-11.4)
ORG-20599	3.10 ± 0.54	86.4	6.21 ± 0.42	186.3	120	310	18.6 (16.6-20.6)
AD	0.86 ± 0.19	33.2	1.95 ± 0.29	49.9	1710	3120	44.0 (30.2-58.8)

$E_{1\ \mu M}$ values represent the mean normalized tonic current responses of drug at 1 μM concentration co-applied with 1 μM GABA. GABA 1 μM tonic current: 0.66 ± 0.22 pA/pF, 19.6 pA.
$E_{3\ \mu M}$ values represent the mean normalized tonic current responses of drug at 3 μM concentration co-applied with 1 μM GABA. GABA 3 μM tonic current: 1.35 ± 0.23 pA/pF, 33.5 pA.
EF values represent the effective functional concentration of drug (nM) required to double or triple the 1 μM GABA response.
6-Hz test ED_{50} values represent the dose in milligrams per kilogram that protected 50% of animals in the 6-Hz seizure stimulation test. 95% confidence intervals are listed in parenthesis, according to a normal distribution.

Differences in neursosteroid sensitivity could be related to alterations in $GABA_A$ receptors as the primary targets of neurosteroid anticonvulsants, as previously determined (see section IV.1.8 and **Figure 14**). We quantified sex-specific dose-dependent responses of neurosteroid in anticonvulsant control of 6-Hz seizures (**Figure 27**). Neurosteroid sensitivity in seizure protection was measured in terms of drug potency (ED_{50}) between male and female WT mice. AP dose-response curves revealed no significant differences between male (ED_{50}: 4.0 ± 0.4 mg/kg) and female (ED_{50}: 4.5 ± 0.5) potency ($p = 0.4450$). Conversely, GNX treatment prior to stimulation displayed greater sensitivity in female (ED_{50}: 1.5 ± 0.1) mice than in males (ED_{50}: 2.9 ± 0.4; $p = 0.0032$). We observed similar incidence of 6-Hz seizure protection by androstane AD in both males and females as denoted by potency (males: 47.8 ± 4.1 mg/kg, females: 44.0 ± 2.2 mg/kg; $p = 0.4248$, $n = 10$ animals per group). The overall potency of AD was significantly less than pregnane steroid counterparts, as expected from its less potent effect in tonic current modulation (**Figure 23**) and relatively lower affinity for $GABA_A$ receptors (Reddy and Jian, 2010).

Previous study of motor toxicity with 3α,5α-pregnane steroids demonstrated that motor impairment is relatively low at doses that confer seizure protection (Kokate et al., 1994). In addition, the TD_{50} (95% confidence limit) value for androstanediol in males was previously determined to be 148 (96-226) mg/kg (s.c.) (Reddy and Jian, 2010). Reports have demonstrated that treatment with 5α-reduced compounds produce lower incidence of motor toxicity compared to the 5β-epimers (Kokate et al., 1994). This is relevant for the protective index (TD_{50}/ED_{50}) of neurosteroids in their clinical use for antiseizure therapy, however subcutaneous treatment of the maximum efficacy dose did not exhibit a plateau or full motor toxicity in our experiments. The experimental rate of absorption for subcutaneous injection of steroid was relatively slower, and therefore we did not observe significant toxicity in animals at maximally effective doses for anticonvulsant effect. A previous study examination of loss of righting reflex as a measure of AP's anesthetic effect found minimal impairment at 10 mg/kg (s.c.) in WT male and female mice (Reddy and Zeng, 2007). Therefore, the ED_{100} of neurosteroid may be representative of binding and modulation of a more specific $GABA_A$ receptor population.

To better understand the structure-activity of neurosteroids at extrasynaptic receptors, we sought to correlate the anticonvulsant responses of neurosteroids and the positive modulation of tonic

current inhibition. We tabulated the previously described $E_{1\ \mu M}$ and $E_{3\ \mu M}$ values for neurosteroid modulation of tonic current, the effective functional concentrations required for the doubling ($EF_{2\text{-fold GABA}}$) or tripling ($EF_{3\text{-fold GABA}}$) of the baseline 1 μM GABA response, and the potency of seizure protection in the 6-Hz model (**Table 6**). The $EF_{2\text{-fold GABA}}$ concentration for ALFX was significantly greater than responses for allosteric AP and GNX, denoting detriment of the C11 structural modification to extrasynaptic receptor efficacy. Interestingly, the ORG-20599 compound had a high extrasynaptic receptor efficacy comparable to that of AP, however the anticonvulsant potency was highly reduced. This may be due in part to the pharmacokinetic differences of the compound's access to the brain subsequent to injection. GNX displayed the highest potency and had slightly lower tonic current efficacy than AP.

Overall, these results suggest that tonic current function is highly dependent on δ-subunit-containing extrasynaptic receptors. These receptors have strong affinity for neurosteroids which are able to robustly enhance tonic currents leading to anticonvulsant effects mostly via network GABAergic inhibition.

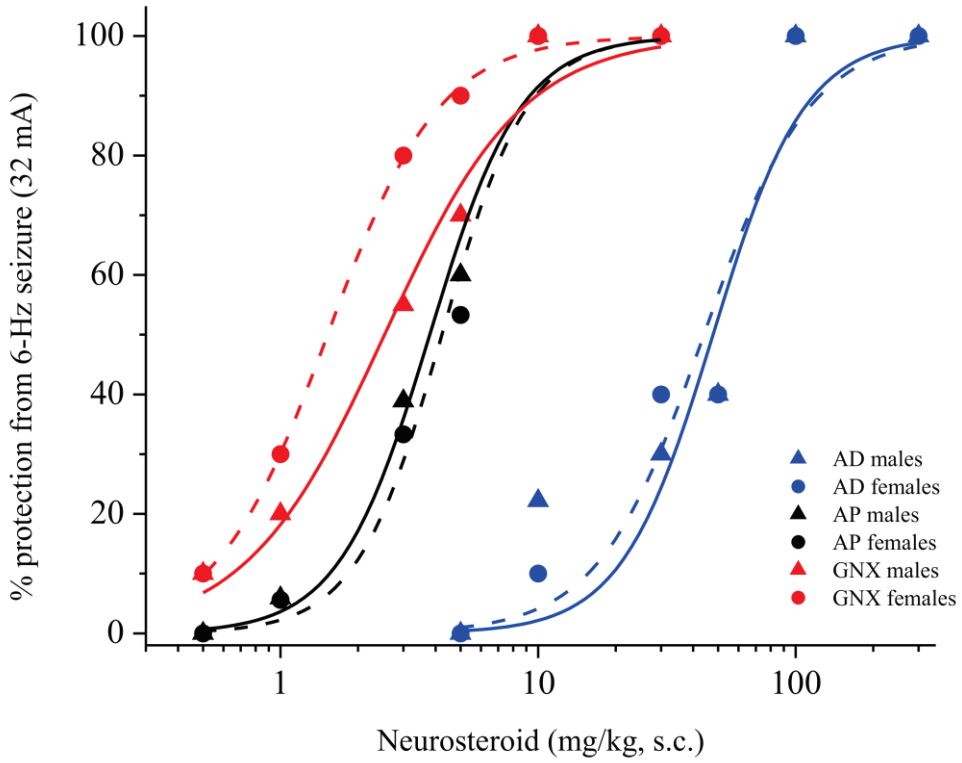


Figure 27. Neurosteroid structure-activity between male and female WT mice in the 6-Hz seizure model. Dose-dependent neurosteroid anticonvulsant responses *in vivo* from WT male and female mice. Neurosteroid was administered subcutaneously 15 min. prior to corneal stimulation. The endogenous neurosteroids AD (*blue*) and AP (*black*), and the synthetic neurosteroid GNX (*red*) were compared in terms of antiseizure potency within WT males (*triangle symbols and dashed-lines*) and females (*circle symbols and solid lines*). AD and AP had similar profiles across gender, whereas females had greater sensitivity to GNX protection than males. 6-Hz potency ED_{50} values represent the dose in milligrams per kilogram that protected 50% of animals in the 6-Hz seizure test. n = 8 – 10 mice per group.

IV.3 Alterations in Hippocampus Epileptogenesis and Behavior in Mice with Deletion of δ-subunit-containing $GABA_A$ Receptors

IV.3.1 Accelerated kindling epileptogenesis in germline δKO mice

To investigate the role of the δ-subunit in suceptibility to epileptogenesis, we studied the development of hippocampus kindling in germline δKO mice. The kindling model of epileptogenesis is a robust model of complex partial seizures (Goddard et al., 1969; Löscher, 2002; McNamara et al., 1992). We have already demonstrated in the rapid kindling model that naïve δKO mice exhibit faster rate of kindling and lower threshold for eliciting electrographic afterdischarge than WT mice (**Figure 13**). We sought to compare WT and δKO mice in the regular hippocampus kindling model to determine differences in epileptogenesis. Mice were implanted with an electrode in the right hippocampus and allowed to recover from surgery (see Methods section III.5). Next, mice were stimulated with increasing current amplitudes in order to determine the afterdischarge threshold, which is the synchronous electrographic waveform upon stimulation of the hippocampus (**Figure 28**). Stimulation resulted in immediate electrographic activity, monitored in real-time. For kindling epileptogenesis of WT and δKO mice, we used the regular kindling model of epileptogenesis, in which stimulations were delivered once per day. Subsequent behavioral seizures were scored according to Racine's scale as modified for the mouse: stage 0, no response or behavioral arrest; stage 1, chewing or facial twitches; stage 2, chewing and head nodding; stage 3, forelimb clonus; stage 4, bilateral forelimb clonus and rearing; stage 5, rearing and falling (Racine, 1972). Consistent stage 5 motor seizures represent a fully kindled state and behavioral end-point for the experiment. Three key parameters were assessed as criteria for seizure susceptibility: 1) Afterdischarge threshold current for electrographic seizures, 2) stimulation-induced electrographic afterdischarge duration, and 3) behavioral seizure intensity over number of stimulations.

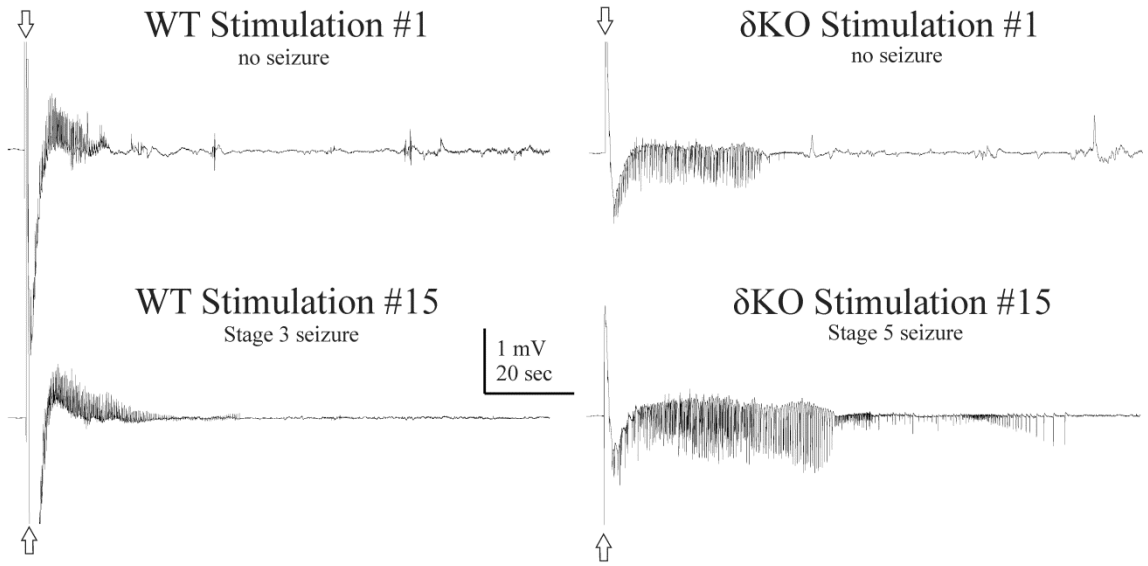


Figure 28. Electrographic afterdischarge of brain activity recorded from hippocampus kindling epileptogenesis mice. Representative electrographic afterdischarge recordings from hippocampal, bipolar electrode in WT or δKO female mice immediately after kindling stimulation. The arrows denote the stimulus artifact occuring at the instant of stimulation and switching to recording mode. Synchronous network activity is apparent immediately after stimulation as the electrical afterdischarge. Current stimulation eliciting afterdischarge of at least 5 sec was determined on day 0 of kindling. Daily kindling stimulation was given at 125% afterdischarge threshold using an isolated pulse stimulator. Stimulations were delivered daily until stage 5 behavioral seizures were consistently achieved, signifying a fully kindled state. The top row depicts traces acquired from animals during the first stimulation in which no overt motor seizures followed from the afterdischarge. The bottom row depicts traces from the same animals during their 15^{th} stimulation, in which the WT mouse displayed stage 3 seizures, and the δKO mouse displayed stage 5 fully-kindled seizures subsequent to the afterdischarge. Properties of afterdischarge and behavioral seizure were simultaneously recorded for each stimulation. Mean group data are reported in subsequent figures.

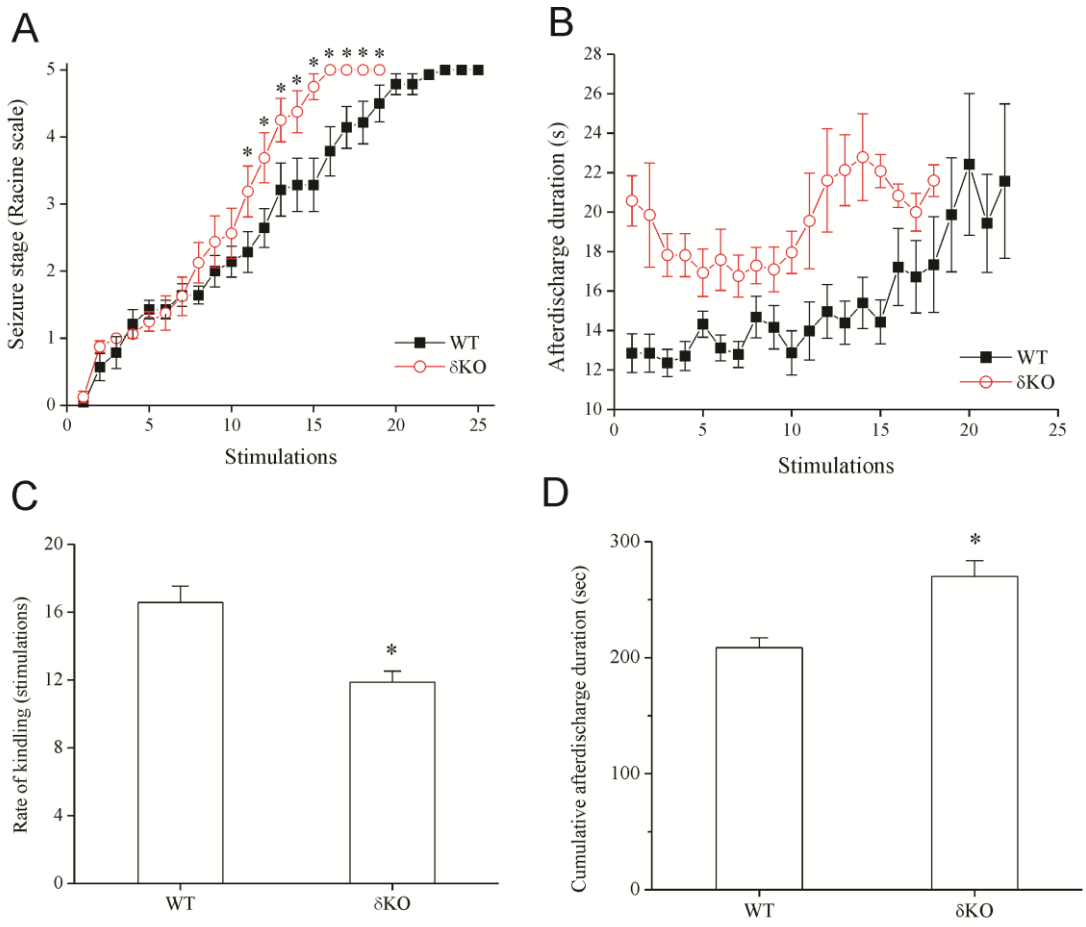


Figure 29. Accelerated epileptogenesis in δKO mice in the hippocampus kindling model of epileptogenesis. ***A***, Kindling progression of behavioral seizure development in female WT and δKO mice as average seizure stage on stimulation. δKO mice had lower mean seizure stage than WT at the corresponding stimulation session. Single asterisks indicate that the mean value is significantly different between groups ($p < 0.05$ vs. WT). ***B***, Afterdischarge duration is prolonged in δKO animals compared to WT. ***C***, Mean overall rate of kindling development (number of stimulations for stage 4/5 seizures) was significantly faster in δKO mice. ***D***, Mean cumulative afterdischarge duration was significantly greater in δKO mice. Values represent the mean ± SEM (* $p < 0.05$; n = 14-16 mice per group).

The progression of electrographic afterdischarge activity, behavioral seizures, and the rate of kindling were recorded as main indices of epileptogenesis. The mean seizure stage and afterdischarge duration for each genotype were plotted according to the stimulation number for direct comparison of kindling progression (**Figure 29A-B**). The δKO mice exhibited accelerated kindling development as demonstrated by fewer stimulations required to elicit behavioral seizures and lower mean seizure stage at the corresponding stimulation session. The rate of kindling was determined as the total number of stimulations required to elicit stage 4/5 seizure (**Figure 29C**). Electrographic activity differences between genotypes were also evident, as δKO mice had a significantly prolonged afterdischarge duration compared to WT controls. The cumulative afterdischarge duration was also significantly greater in δKO mice, indicating that compared with WT mice, the knockouts had prolonged synchronous activity over a substantially shorter period of time (**Figure 29D**).

There has been previous evidence to suggest inherent sex-specific differences in seizure susceptibility (Galanopoulou, 2008ab, Reddy, 2009b), although this aspect of epileptogenesis is not widely investigated. To determine a possible role of δ-subunit in the sex-dependent effects on epileptogenesis, we analyzed seizure progression profiles in male and female δKO mice (**Figure 30**). Among the δKO animals, we observed that male mice exhibited more rapid and intense epileptogenesis than female mice. However, WT male mice similarly displayed an increased rate and propensity of seizure than WT females. Male mice experienced faster kindling rate and exhibited generally longer electrographic afterdischarges throughout the progression of epileptogenesis.

Furthermore, accelerated epileptogenesis was evident in both male and female δKO mice compared to WT counterparts of same gender. Male δKO mice displayed significantly greater mean seizure stage for stimulations 8-16 than male WT mice ($p < 0.05$, $n = 16$ animals per group). Similarly, female δKO mice displayed signficantly greater mean seizure stage for stimulations 11-17 ($p < 0.05$, $n = 14$-16 animals per group). These stimulations represent late-stage epileptogenesis responses (stage 4-5 seizures) for δKO animals, but mid-kindling responses (stage 2-3 seizures) for WT animals. Overall, mean number of stimulations required to reach stage 5 seizures was significantly lower in δKO animals (**Figure 30C**).

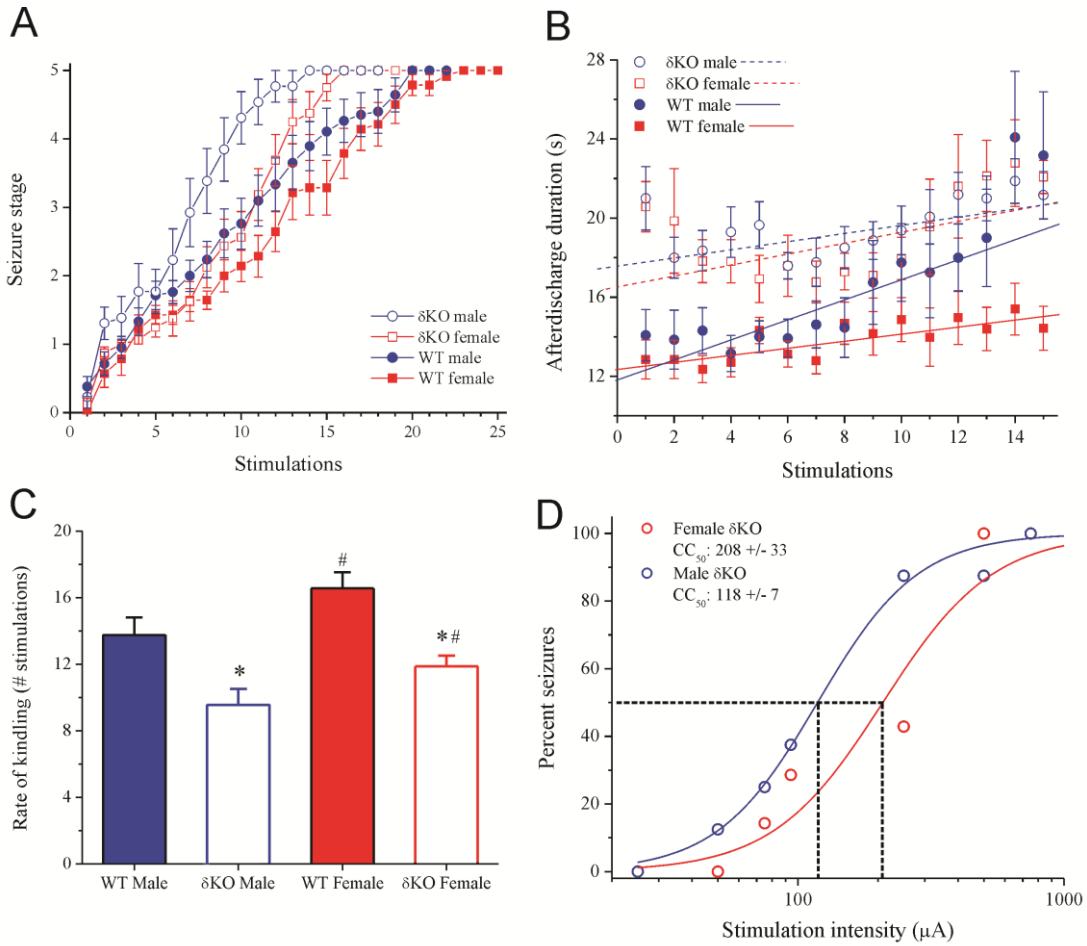


Figure 30. Male mice exhibit more rapid and intense epileptogenesis than female mice, independent of genotype. ***A***, Gender and genotype comparison of kindling epileptogenesis progression. ***B***, Afterdischarge duration for WT and δKO. Knockouts display prolonged afterdischarge diuration vs. WT. Males display prolonged afterdischarge duration vs. females. ***C***, Mean number of stimulations required to reach a fully kindled state (stage 5 seizure) in mice (*p < 0.05 vs. WT of same gender, #p < 0.05 vs. males of same genotype). ***D***, Afterdischarge threshold sensitivity expressed by stimulation-response curves from δKO male and female 30 days after kindling. n = 8 – 16 mice per group.

To investigate retention of kindling hyperexcitability, the afterdischarge threshold sensitivity was determined in δKO mice thirty days after attaining the fully kindled state. Our aim was to explore the sensitivity to convulsant stimuli in which epilepsy-prone circuits are already established within the brain. In analysis of stimulation response curves due to increasing current intensity (μA), we found that male mice possessed greater sensitivity than females in the exacerbation of seizures (**Figure 30D**). The CC_{50} (convuslant current) value was determined for each group, which was the current required to elicit stage 5 seizures in 50% of animals 30 days after kindling. The CC_{50} value for female δKO mice was 208 ± 33 μA, whereas the CC_{50} for male δKOs was 118 ± 7 μA ($p = 0.0184$, $n = 8$ -12 mice per group). These data signify that male δKO mice have a higher seizure retention rate and greater sensitivity than females. Furthermore, the hyperexcitability attributed to deletion of the δ-subunit may be exacerbated disparately, according to sex-specific characteristics in the brain. This may be attributed to distinctive neuroendocrine phyisiologies that may influence the balance of inhbition and excitation within the brain. Our hypothesis is consistent with previous reports, suggesting that $GABA_A$ receptor expression may contribute to sex-specific alterations in the brain that may be significant to epileptogenic activity (Galanapoulou, 2008ab).

IV.3.2 Neurosteroid disease-modification of kindling epileptogenesis

Retarding of kindling epileptogenesis by AP is diminished in δKO female mice

We sought to ascertain the *in vivo* role of neurosteroids as disease-modifying agents in the progression of epileptogenesis in δ-subunit knockout. Neurosteroid exposure produces an anticonvulsant, protective function in fully kindled animals (Reddy et al., 2012), but we sought to investigate neurosteroid-mediated prolongation of the hippocampal kindling epileptogenesis process. We administered AP (0.5 mg/kg, s.c.) to WT and δKO female mice prior to each daily stimulation in the regular kindling paradigm. AP significantly impeded the kindling progression in both WT and δKO mice (**Figure 31**). However, we observed that the neurosteroid-induced retardation of epileptogenesis was diminished in δKO mice. WT mice experienced a slower rate of kindling (stage/stimulation or slope of progression) than δKO mice in the AP treatment group. Afterdischarge duration was similar between genotypes. All mice reached stage 5 seizures within 30 stimulations, signifying an inability of this relatively low dose of AP to fully oppose the epileptogenic activity. In comparing the neurosteroid-mediated delay between control and AP treatment groups, we found the percent change in rate of kindling to be similar between WT (34.6% retardation of slope by AP) and δKO (39.2% retardation of slope by AP).

To investigate the rate of kindling more critically, we then parsed the rate slope into two segments: stimulation 1 to 14 and stimulation 14 to end point stage 5 seizures (**Figure 31D**). The rate of kindling for the first 14 stimulations ($\leq$ stage 2 seizures in each strain) were not significantly different between WT and δKO in the AP treatment group (WT: 0.09 ± 0.01 stage/stimulation, δKO: 0.12 ± 0.03 stage/stimulation). However, stimulation 14 to the fully kindled state revealed a significantly greater rate of kindling for δKO mice (0.36 ± 0.07 stage/stimulation) compared with WT (0.18 ± 0.02 stage/stimulation, $p = 0.0330$; $n = 6$ mice per group). Therefore, late-stage kindling progression (stage 14+) in δKO animals displayed more accelerated epileptogenesis during neurosteroid AP treatment.

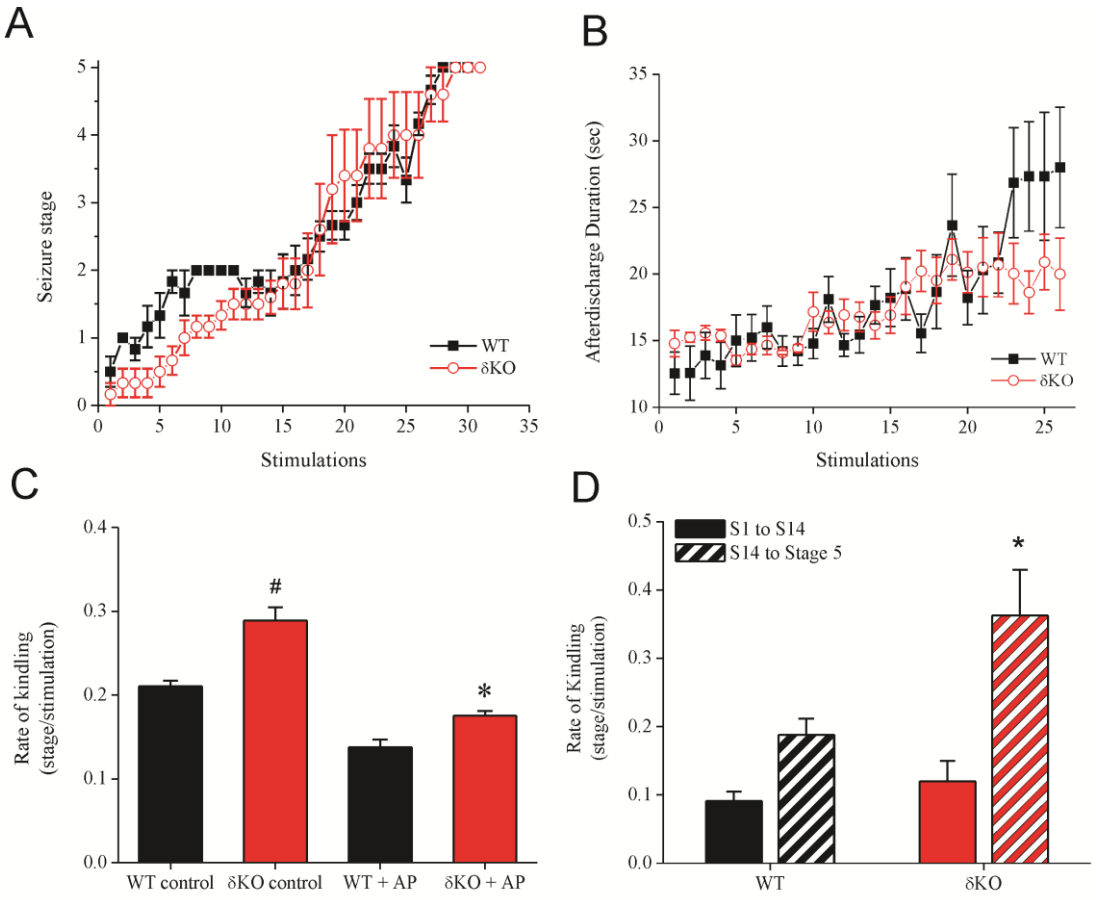


Figure 31. Allopregnanolone-induced retardation of epileptogenesis is diminished in female δKO mice. ***A***, Kindling progression in AP-treated female WT and δKO mice. ***B***, Treatment with AP reduced the afterdischarge duration in δKO mice to levels comparable to WT mice. AP was administered (0.5 mg/kg, s.c.) 15 minutes before each daily kindling stimulation. ***C***, Slope parameter for rate of kindling epileptogenesis in control and AP-treated mice (*p < 0.01 vs. WT + AP; # p < 0.001 vs. WT control). ***D***, Rate of kindling for AP –treated mice for stimulations 1-14 and stimulation 14 to stage 5 seizure. (*p < 0.05 vs. WT S14 to Stage 5). n = 6 -13 mice per group.

IV.3.3 Seizure activity-induced mossy fiber sprouting is enhanced in δKO mice

To explore structural changes associated with epileptogenesis, mossy fiber sprouting was analyzed in WT and δKO mice that experienced regular kindling progression of epileptic seizures (**Figure 32**). Timm staining is a technique of histochemical labeling of the synaptic terminals of mossy fiber axons projecting from DGGCs (Cavazos et al., 1991). This histochemical reaction is possible due to the high zinc granule content in mossy fibers. Previous electroconvulsive kindling models in rodents have reported mossy fiber sprouting into the granule layer and greater zinc granule accumulation in the hilus (Gombos et al., 1999; Watanabe et al,. 1996). We processed Timm-staining of hippocampus mossy fibers in the dentate gyrus in sections from control and kindled animals. Rather than using non-parametric Timm index scoring criteria, we analyzed the histology with densitometry software (see Methods section III.3). We used this approach to avoid observational bias, and in addition, the observer was blind to the tissue genotype and condition in densitometric analysis. Average normalized stain intensities from dentate hilus and the granule layer were acquired from WT and δKO mice experiencing sham control or kindling conditions. The mossy fibers were evident in projection to the CA3 region by the darkly stained zinc granules originating in the dentate hilus (**Figure 32A**). Staining was significantly denser in the hilus than the granule layer in all conditions. Both kindled genotypes displayed increased histochemical density in the hilus. Furthermore, the δKO mice subject to kindling had significantly greater staining intensity in the granule cell layer than seizure-naïve δKO control animals, indicative of seizure activity-induced mossy fiber sprouting. In analysis of WT granule layer staining, little or no sprouting could be observed, and control mice did not display signficantly different densitometry scores than WT kindled mice. We did not observe any differences to granule cell density, cell number, or hilus volume, consistent with previous report (Watanabe et al., 1996). Interestingly, the axonal projections of CA1 and CA3 principal neurons also exhibited greater staining density in kindled animals compared with control. This increase in zinc content could be indicative of axonal sprouting brought on by the repeated, convulsive seizures.

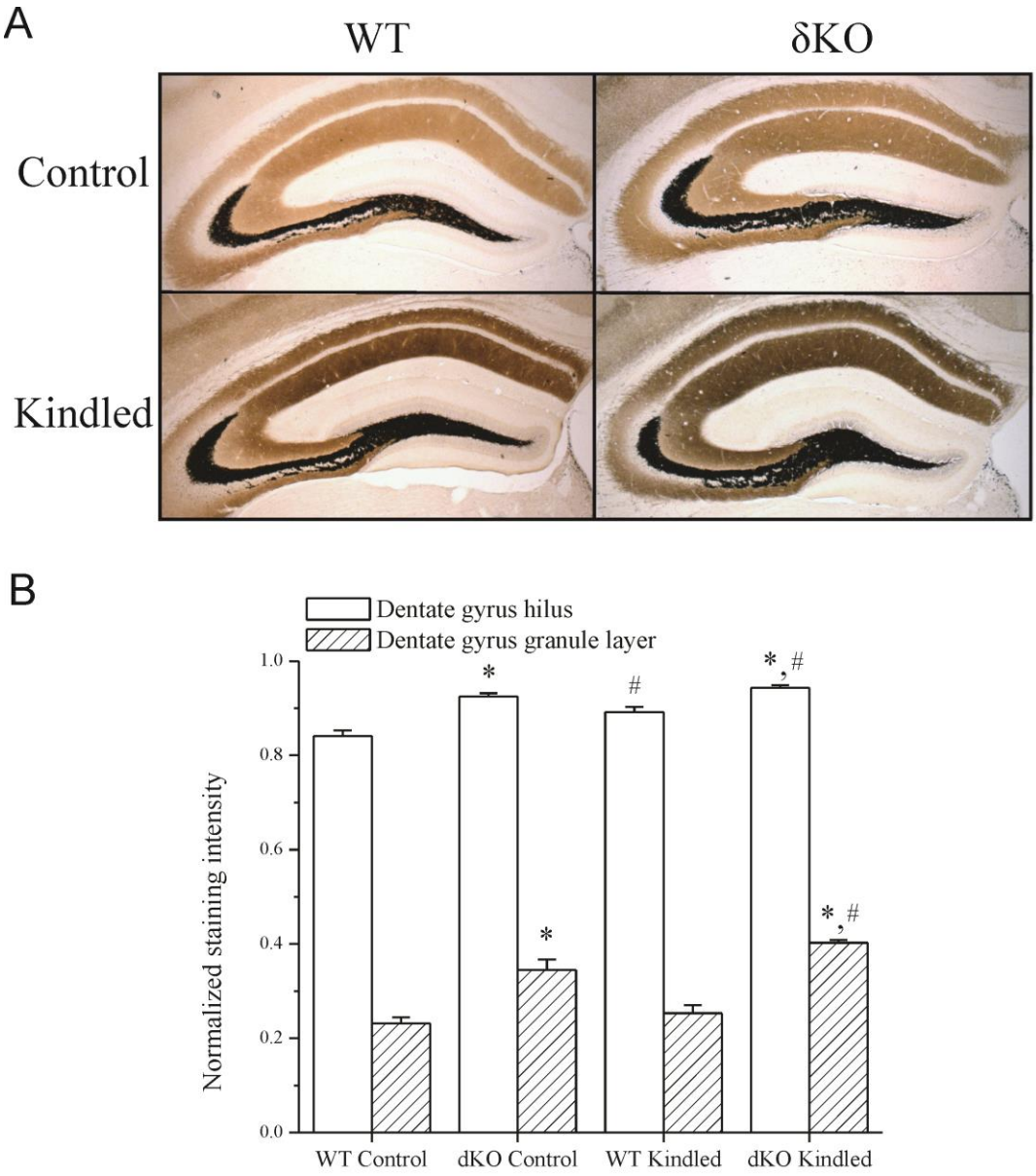


Figure 32. Seizure activity-induced mossy fiber sprouting is enhanced in δKO mice. ***A***, Timm stain histological sections of mouse hippocampus displaying mossy fiber zinc granules in the dentate hilus. ***B***, Averaged densitometry of dentate hilus or granule layer stain intensity within the dentate gyrus. Each intensity score was normalized to length of dentate gyrus. Scores compiled for 3 animals in each group (* $p < 0.05$ vs. WT of same condition, # $p < 0.05$ vs. control of same genotype, $n = 9$ sections per group, independent t-test). Sections were analyzed with the experimenter blinded to the genotype and condition.

IV.3.4 Pentylenetetrazol chemoconvulsant seizure suceptibility

To comparitively determine seizure susceptibility with an acute measure of epileptic seizure onset, we tested the sensitivity of WT and δKO mice to the chemoconvulsant pentylenetetrazol (PTZ) (**Figure 33**). PTZ is a $GABA_A$ receptor antagonist thought to block the channel pore, similar to the action of picrotoxin (Squires et al., 1984), although the mechanism of action resulting in systemic convulsive activity is not well understood (Papp et al., 1987). Previosuly, PTZ low-dose hypoactivity was examined in conjunciton with GNX pro-absence effects on δKO (Mihalek et al., 1999), but it has not been extensively studied as a model of generalized seizures in germline δKO mice.

The convulsant sensitivity of PTZ varies according to the mouse strain being tested (Kosobud and Crabbe, 1990). Therefore, we first determined the convulsant sensitivity of PTZ in mice. Seizure phenotype was scored by the latency to seizures in behavioral response to subcutaneous PTZ injection (see Methods section III.5). Latency to jerking spasms, latency to generalized clonus, and latency to tonic-clonic seizures were recorded for each mouse. A single score of 0 denotes no incidence of behavioral seizure, whereas score is directly proportional with seizure severity. Low-dose, 30 mg/kg PTZ resulted in mild hypoactivity onset for all mice several minutes after injection. Jerking spasms and sporadic clonus were observed in animals at 50 mg/kg PTZ. At 75 mg/kg, convulsant activity consistently culminated in tonic-clonic seizures. The 90 mg/kg dose resulted in lethality in all animals in all groups, and we took this represent the maximum convulsant dose (CD_{99}) in both genotypes.

Experimental mice were demarcated into sex-specific groups for analysis of PTZ-induced seizure severity (**Figure 33A**). The δKO mice displayed similar pharmacological profiles of PTZ convulsant sensitivity to WT counterparts. The δKO female mice displayed significantly greater seizure severity than WT at 60 mg/kg PTZ. There were no other significant difference in seizure score severity between δKO and WT animals. However, at 60-90 mg/kg PTZ, we observed higher seizure severity and toxicity in female mice compared with males. This sex-specific PTZ susceptibility was evident in both WT and δKO females when compared to male gentoype equivalents.

We determined the percent clonic seizure and percent tonic-clonic seizure occurrence in WT and δKO mice as parallel measures of the dose-dependent response of PTZ-induced seizures (**Figure 33B,C**). A clearly defined, significant effect of gender was observed in the appearance of generalized clonic seizures as females exhibited higher clonus occurrence. There were no significant differences in occurrence of clonic seizures between WT and δKO mice at higher concentrations (60 – 90 mg/kg). In addition, females experienced higher incidence of tonic-clonic seizures than male mice.

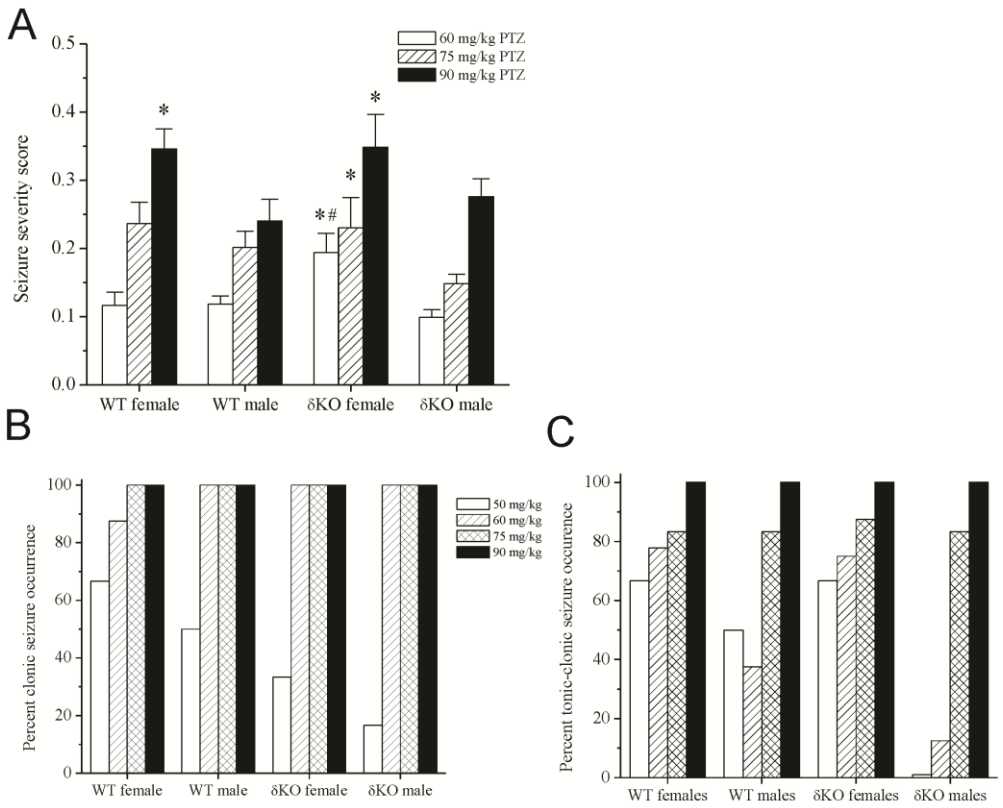


Figure 33. PTZ-induced seizure severity in germline δKO mice. ***A***, Seizure severity scores from WT and δKO male and female mice. Latency to jerking spasms, latency to clonus, and latency to tonic-clonic seizures were scored based on a weighted mechanisms to produce seizure severity. WT and δKO mice displayed similar seizure severity at higher convulsant doses of PTZ (75-90 mg/kg). However, sex-specific, significant differences were evident in seizures severity of WT mice (* $p < 0.05$ vs. male of same genotype and dose, # $p < 0.05$ vs. WT female at 60 mg/kg, n = 6-8 animals per group). ***B***, Percent clonic seizure occurrence or ***C,*** percent tonic-clonic seizure occurrence in WT and δKO mice in the PTZ (50, 60, 75, and 90 mg/kg, s.c.) test. Each mouse was evaluated for generalized clonic seizing and tonic-clonic hindlimb extension following PTZ injection (n = 6-8 animals per group).

IV.3.5 Developmental phenotype and cognitive behavior in germline δKO mice

Immunohistochemistry staining of δ-subunit in germline δKO mice

To confirm the deletion of δ-subunit in the δKO mice, we processed brain tissue sections with a δ-specific antibody in comparison with WT mice. The δKO sections did not reveal any immuno-reactivity, as represented in the hippocampal sections (**Figure 34**). The thalamus contains high expression of δ-containing receptors (Pirker et al., 2000; Wafford et al., 2009). Thalamic regional expression δ-subunit pattern was also ablated upon knockout.

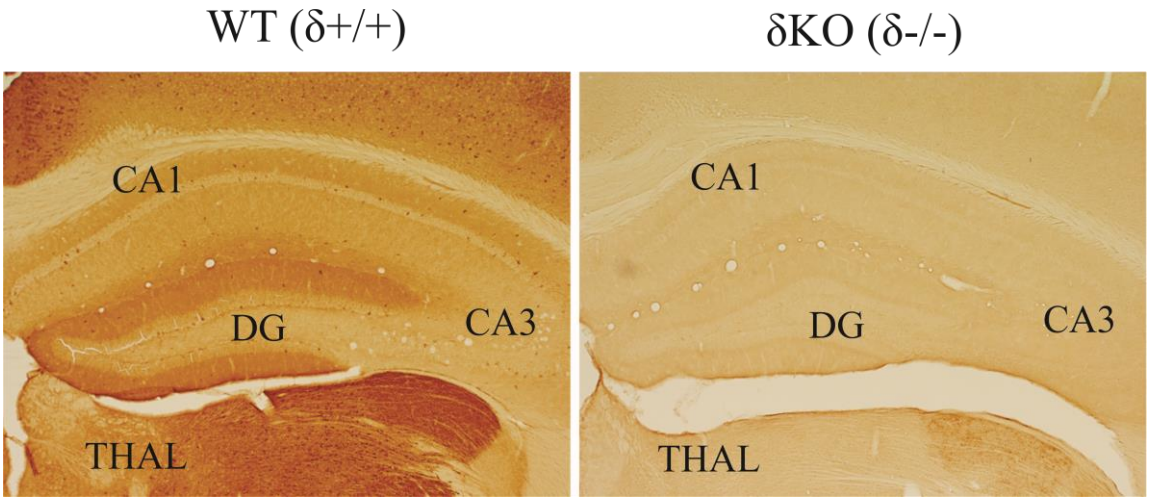


Figure 34. Immunohistological confirmation of δ-subunit deletion in germline δKO mice. $GABA_A$ receptor δ-subunit (*Gabrd*) antibody (1:250) was used for staining the δ-subunit pattern in transverse sections of hippocampus. Immuno-staining was completely absent in sections from δKO mice. In the WT, δ-positive section, strong staining is evident in the dentage gyrus molecular layer and thalamus (THAL). Only background, non-specific staining was evident in the δ-null sections (right image). This non-specific staining was lighter in intensity in smaller titres of antibody staining.

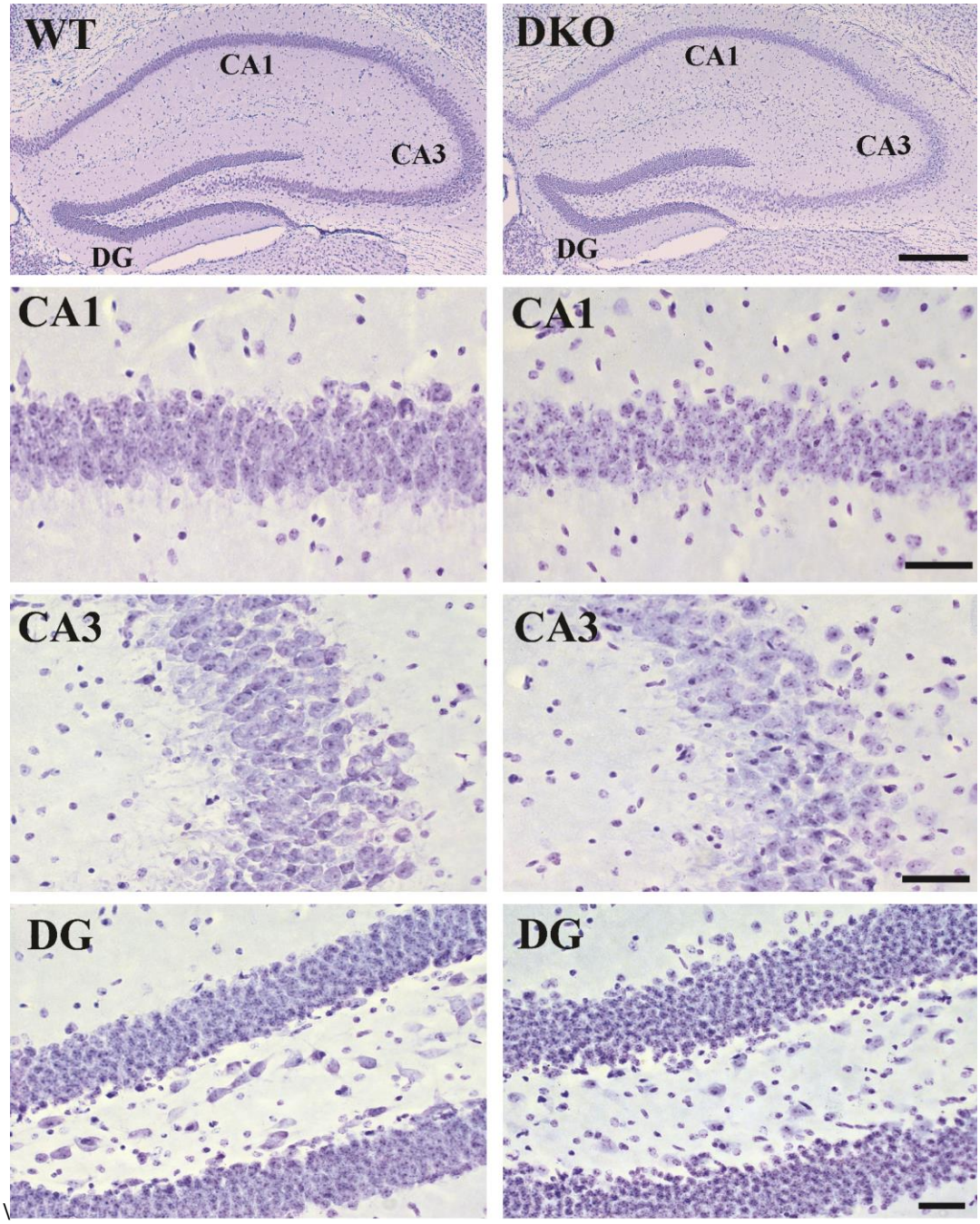


Figure 35. Morphological profile of δKO and WT mouse hippocampus. Nissl-stained sections of WT and δKO mice reveal no overt abnormalities in the cytoarchitecture in the dorsal hippocampus. Nissl-stained neurons within the dorsal hippocampus CA1, CA3, and DG regions were similar in WT and δKO mice. Scale bars: Top two panels, 400 μm; CA1, CA3, and DG panels, 50 μm.

Histological comparison of brain morphology

There have not been any previous reports of histological or anatomical brain differences of germline δKO. We performed cytochemical studies with Nissl-stained δKO and WT mouse brain to ascertain any morphological differences. Histological analysis revealed normal architecture of the ventral and dorsal hippocampus, amygdala, neocortex, and hypothalamic regions in δKO mice. The pyramidal layers at the CA1 and CA3 subfields, and hilar and granule cells of DG in the δKO mouse appeared normal, and were similar to age-matched WT control (**Figure 35**). However, CA3 and DG regions of the dorsal hippocampus had greater dispersion of principal neurons in δKO mice. Overall, hippocampal cell architectual abnormalities were not evident in δKO mice.

Fecundity and development of δKO mice

The key limitation of the germline δKO mouse model is developmental defects that could confound the phenotype. Therefore, neonatal and recently-weaned germline δKO mice were examined to ascertain any developmental impairment in the transgenic strain. Previously reports found that approximately 5% of knockout pups die before weaning, and in addition, knockout breeders produce fewer pups per litter overall compared to WT breeders (Mihalek et al., 1999). Consistent with this finding, we report fewer mice per litter from homozygous knockout breeding pairs ($\delta^{-/-}$) (5.39 ± 0.27, n = 60) than WT breeding pairs ($\delta^{+/+}$) (7.21 ± 0.34, n = 92; p = 0.0002) (**Figure 36A**). An average 25.3 ± 5.3 % of δKO mice were lost prior to weaning, whereas an average of 4.2 ± 2.0 % WT mice died before weaning (**Figure 36B**). Measurements of weight were taken for mice twice a week for 80 days after weaning. In the first 14 days after weaning, male and female δKO mice displayed significantly lower weights than WT counterparts (**Figure 36C**). Our findings are consistent with previously published data that suggests the deletion of the δ-subunit has a critical impact on reproduction, and we uncover further developmental defects of the transgenic δ-subunit mutant.

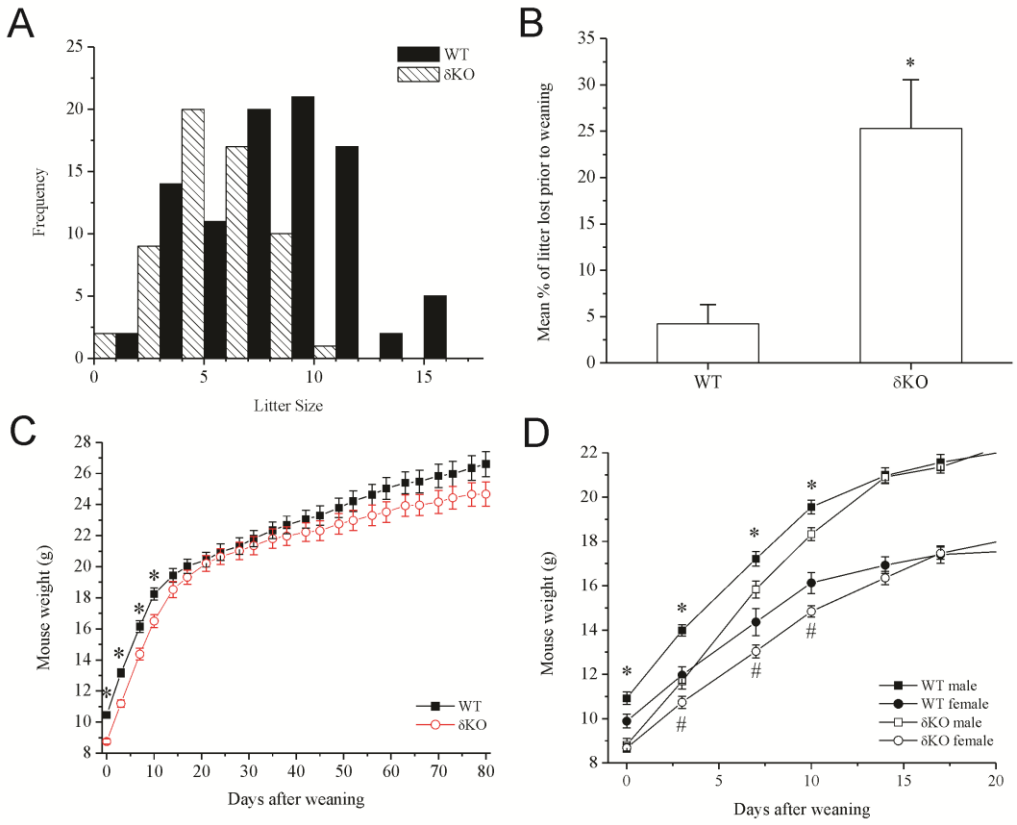


Figure 36. Developmental alterations in germline δKO mice. ***A***, Histogram depiction of the number of pups per litter born to WT or δKO breeding pairs. A left shift in normalized frequency is evident in the δKO group. Mean mice per litter was significantly lower in δKO mice compared with WT mice (see text). ***B***, Mean percentage of pups per litter that died prior to weaning (* $p < 0.05$ vs. WT, n = 60 $\delta^{-/-}$ litters, 92 $\delta^{+/+}$ litters). ***C***, Average mouse weight of WT and δKO pups from 0-80 days after weaning (* $p < 0.05$ vs. δKO, n= 23 δKO mice, n=31 WT mice). ***D***, Sex-specific comparison of weights 0-20 days after weaning for WT and δKO mice. Males exhibited significantly greater weights than females. (* $p < 0.05$ for male WT vs. δKO comparison, # $p < 0.05$ for female WT vs. female δKO comparison). WT and δKO weights normalized 12 days after weaning, and were not significantly different 12-80 days after weaning.

Porsolt forced swim test

To investigate changes in behavioral depression, the Porsolt forced-swim test was performed in both male and female WT and δKO mice (**Figure 37**). The Porsolt test is the accepted paradigm for studying depression in mouse models (Porsolt et al., 1978). Mice were placed in an inescapable cyllinder of water for 6 minutes, and latency to the first immobility and total duration of immobility were measured. A mouse was determined to be immobile when it ceased all active behaviors and remained passively floating or making minimal hind-limb movements necessary to maintain nostrils above the water. WT mice exhibited a lower average latency to first immobility (24.6 ± 1.9 s) than δKO mice overall (60.8 ± 3.2 s), signifying a faster rate of passivity (**Figure 37A**). Mean latency to first immobility for male and female WT mice was 21.9 ± 2.4 s and 26.9 ± 2.8 s, respectively. Latency to first immobility for male and female δKO mice was 59.0 ± 5.3 s and 62.6 ± 3.8 s, respectively (n = 17-19 mice/group). These results signify that male mice had significantly faster rate of first immobility than the female counterparts. Fractional percentage of time spent immobile in the swim test is shown in **Figure 37B**. The δKO mice spent significantly less time immobile than WT mice, with no significant effect of gender (female δKO: 252.7 ± 4.9 s, male δKO: 255.5 ± 5.0s, female WT: 283.9 ± 5.0 s, male WT: 288.4 ± 4.7s). Furthermore, δKO mice had more initiations of active escape behavior than WT mice after initial immobility. These results suggest potential differences in depression behavior in δKO mice due to the constitutive removal of the δ-subunit-containing $GABA_A$ receptors.

Elevated plus-maze test

We used the elevated plus-maze learning task to determine if δ-subunit deficiency produces cognitive deficits in knockout mice (**Figure 38**). Female WT and δKO mice displayed significant differences in the Day 1 transfer latency, however male mice displayed similar times. We did not observe a significant effect of gender, although female mice had a shorter Day 1 transfer latency compared to males among both WT and δKO mice. Day 2 transfer latencies did not display a significant effect regarding either strain or gender. The difference in transfer latency between the acquisition (Day 1) and retention (Day 2) trial was not significantly affected by gender or strain. Therefore, we conclude that baseline conditions of memory consolidation tested by the elevated plus maze are not significantly effected by δ-subunit germline deletion.

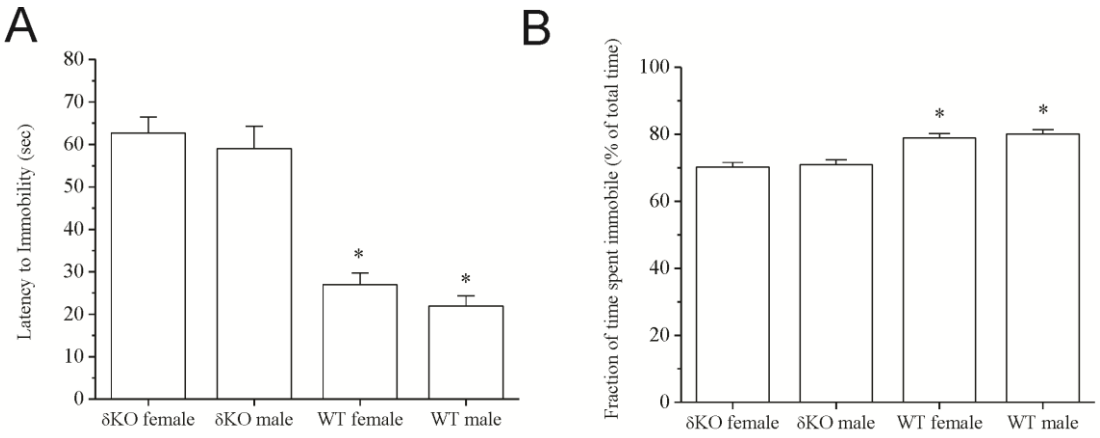


Figure 37. Porsolt forced swim test of depression behavior in δKO mice. ***A***, Quantification of latency to first time of immobility in WT and δKO mice, stratified by sex. ***B***, Fraction of time spent immobile in the 6 minute Porsolt forced swim test, expressed as a percentage for WT and δKO, stratified by sex. All animals were 2 months old. * $p < 0.05$ vs. δKO counterpart of same sex, n = 17 – 19 animals per group.

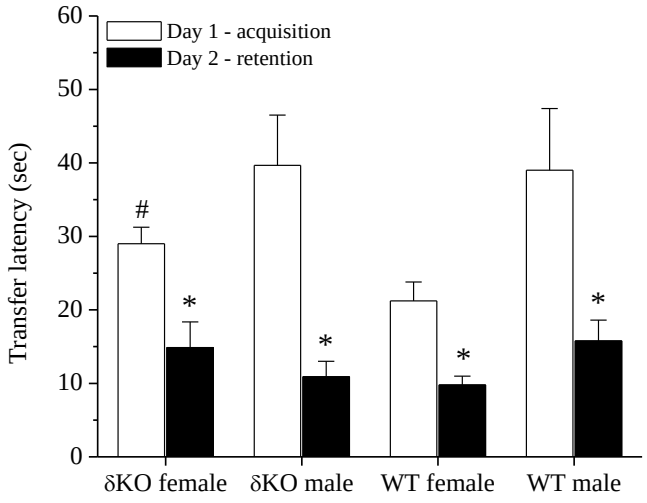


Figure 38. Elevated plus maze test of cognitive behavior in δKO mice. Transfer latency for WT and δKO mice as the time required for a mouse to travel to an enclosed arm of the plus maze when placed at the end of a plus arm. Day 1 (white bars) represents acclamation to the environment and learning the environment. Day 2 (black bars) represents memory retention of the maze. All groups developed mean reduction in transfer latency from Day 1 to Day 2. WT and δKO mice were experimentally naïve on Day 1 and group-matched for age and sex. (* $p < 0.05$ vs. Day 1; # $p < 0.05$ vs. WT female). n = 9 animals per group.

CHAPTER V
DISCUSSION

V.1 Role of δ-subunit Plasticity in Neurosteroid Withdrawal

Perimenstrual-like withdrawal from neurosteroids results in striking increase in extrasynaptic δ-containing $GABA_A$ receptors in the dentate gyrus, a key limbic subregion associated with epilepsy pathology. Up-regulation of δ-subunit confers increased sensitivity to AP potentiation of $GABA_A$ receptor-mediated tonic inhibition in DGGCs. The prominent increase in δ-subunit expression specific to the dentate gyrus exemplifies its "gate-keeping" function in controlling baseline inhibition and network inputs in the hippocampus (Coulter and Carlson, 2007). These findings provide a molecular mechanism for enhanced anticonvulsant activity of neurosteroids in perimenstrual models of catamenial epilepsy and thereby strengthen the rationale for neurosteroid therapy of catamenial epilepsy.

We observed significant up-regulation of δ-subunit expression in the hippocampus during perimenstrual-like NSW, confirming the role of extrasynaptic $GABA_A$ receptors in response to fluctuating neurosteroid levels in the brain. This novel observation is consistent with the ovarian cycle-linked regulation of the plasticity and function of extrasynaptic $GABA_A$ receptors (Reddy et al., 2012; Wu et al., 2013). While other regions within the hippocampus had marginally increased expression of δ-subunit, the withdrawal-induced δ levels and membrane surface insertion were directed most significantly within the dentate gyrus. This in turn resulted in functional increase to neurosteroid potentiation of extrasynaptic receptors. Previous reports of NSW-induced up-regulation of $GABA_A$ receptors have focused on expressional changes within CA1 pyramidal neurons (Smith et al., 1998b; Smith et al., 2007; Sundstrom-Poromaa et al., 2002). NSW-induced increase to δ-subunit has not previously been shown within the dentate gyrus. Our findings of δ-specific perimenstrual up-regulation in DGGCs confirm a functional role for $\alpha_4\beta\delta$ receptors in modulating excitability in catamenial epilepsy.

The mechanisms underlying perimenstrual δ-subunit plasticity remain unclear. The early growth response factor-3 pathway is implicated in NSW-induced α_4 subunit up-regulation and this has been found to occur independently of any PR interaction (Gangisetty and Reddy, 2010). The up-regulation of δ-subunit was also evident in PR knockout mice experiencing NSW. Thus, it is

likely that the δ-subunit plasticity is neurosteroid-dependent and not due to PR activation by progesterone in the perimenstrual model. It is likely that cellular machinery involved in protein and receptor trafficking may have an important role in the expression of $GABA_A$ receptor subunits (Jacob et al. 2008).

AP was utilized as a prototype neurosteroid to study the function of $GABA_A$ receptors in the perimenstrual model. Other neurosteroids could have a role in modulating neuronal excitability (Tuveri et al., 2008; Reddy and Ramanathan, 2012), but the clear correlation between cyclic progesterone and perimenstrual seizure susceptibility led us to hypothesize that NSW and subsequent sensitivity to AP is strongly mediated by δ-containing receptors (Reddy, 2009ab). Finasteride inhibition of 5α-reductase results in decreased AP synthesis in mice (Reddy et al., 2012). This directed decline in AP was employed to determine the basis for neurosteroid sensitivity similar to that occurring in women experiencing perimenstrual withdrawal.

Consistent with previous studies of α_4,δ-containing, recombinant receptors (Brown et al., 2002), our findings in native DGGCs indicate a significant increase in receptor function by AP in the withdrawal model. Physiological concentrations of AP (100-300 nM) produced significantly greater $GABA_A$ receptor current modulation in NSW compared to control mice (**Figure 9,10**). Furthermore, δKO DGGCs did not display enhanced sensitivity to AP upon withdrawal, suggesting that NSW-induced functional changes are δ-directed. This signifies a key role by extrasynaptic, δ-containing receptors in response to endogenous neurosteroids in the dentate gyrus during withdrawal despite a pathological susceptibility to seizures (Stell et al., 2003).

The up-regulation of δ-subunit was substantial enough to allow for a detectable change in AP potentiation of whole-cell $GABA_A$ receptor current in DGGCs but not in CA1PCs. Although we observed significant increases to δ-subunit expression in CA1, this did not result in increased AP potentiation of $GABA_A$ receptor current within these neurons. It has been previously reported that δ-containing receptors do not contribute to neurosteroid sensitivity or inhibitory tonic current within the CA1 (Stell et al., 2003; Glykys et al., 2008). Within male mice under control conditions, tonic inhibition in CA1 neurons is primarily modulated by $\alpha_5\beta\gamma_2$ receptors (Glykys et al., 2008). We found that NSW does not convey functional increase to extrasynaptic current in CA1, as demonstrated with δ-sensitive pharmacology (**Figure 9B,F**). Membrane surface

expression of δ-containing receptors is profuse within dentate gyrus but is sparse within the CA1, CA2, and CA3 regions (Pirker et al., 2000; Peng et al., 2002). We previously reported the estrous cycle-dependent δ-subunit plasticity and function were specific to DG and not to CA1 neurons (Wu et al., 2013). The large difference in AP modulation between DGGC and CA1 neurons suggests region-specific plasticity of $GABA_A$ receptors and neurosteroid sensitivity in this perimenstrual-like model of epilepsy.

In the NSW model, we investigated the tonic current function of extrasynaptic $GABA_A$ receptors in DGGCs. Low, extracellular GABA (0.3 – 1.0 μM) produced greater tonic current in NSW condition than control. The AP potentiation of tonic current in withdrawal DGGCs was also significantly greater than control DGGCs. Within δKO DGGCs, the substantial reduction of tonic current typical for the knockout model did not revert to higher levels upon NSW, confirming that withdrawal-associated potentiation of tonic current is δ-mediated. We also explored THIP modulation of tonic current and found that NSW DGGCs had enhanced tonic current at THIP concentrations reported to be δ-subunit selective (Meera et al., 2011). These findings signify enhancement of neurosteroid sensitivity to promote extrasynaptic receptor function and defines a functional role of δ-sensitive agents to modify network inhibition of the hippocampus.

Up-regulation of δ-subunit expression may affect postsynaptic function. In the NSW model, $GABA_A$ receptor mIPSC properties were unaltered in DGGCs. However, in response to AP, mIPSCs displayed increased amplitude and prolonged decay kinetics. We did not observe any similar AP-sensitivity changes to mIPSC properties in the δKO mouse upon withdrawal. In fact, decay kinetics were modestly reduced in δKO DGGCs during AP application (**Table 2**). Therefore, withdrawal-induced increases in δ levels confer higher synaptic sensitivity to neurosteroids. Our findings are consistent with previous reports of AP-modulated changes in postsynaptic function (Sun et al., 2007). Our results show that AP only potentiated mIPSC amplitude from NSW but not from control cells. Neurosteroids prolong the kinetics of synaptic $GABA_A$ receptor channels, resultant from longer channel gating time in open configuration (Bianchi and Macdonald, 2003). Due to heightened neurosteroid sensitivity in NSW, there is preferential enhancement of the low-efficacy state during neurosteroid binding, thereby contributing to the AP-sensitive phasic currents gated by GABA. We found this modulation of

phasic current to be neurosteroid- and δ-subunit-dependent. mIPSCs from δ-knockout mice DGGCs have a faster decay time constant (Mihalek et al., 1999), and neurosteroid modulation of synaptic currents is reduced (Spigelman et al., 2003). We did not detect changes to subunits in the hippocampus primarily assembled at the synapse (α_1, α_2, β_2, γ_2). Therefore, it is unlikely that the increase of α_4 and δ subunits promotes compensational changes to other subunits in the withdrawal model. Moreover, previous studies suggest that $\alpha_4\beta\gamma_2$ receptors exhibit faster desensitization than $\alpha_4\beta\delta$ but exhibit slower decay kinetics (Brown et al., 2002; Smith and Gong, 2005). Overall, these results expand on previous findings by presenting a novel model in which strong δ-up-regulation promotes neurosteroid sensitivity on phasic receptors.

Despite increased awareness of neurosteroids, the molecular pathophysiology of catamenial epilepsy remains unclear. A decline in neurosteroid in the brain and subsequent reduction of $GABA_A$ receptor-mediated inhibition can create an environment of hyperexcitability within the limbic network. Mice that experience inhibition of AP synthesis show enhanced susceptibility to limbic epileptogenesis (Reddy, 2009b; Reddy et al., 2012). We induced neurosteroid withdrawal in an *in vivo* rapid kindling paradigm to explore acute epileptogenesis during the dynamic $GABA_A$ receptor plasticity. Despite up-regulation of extrasynaptic receptors, wildtype mice undergoing NSW experienced heightened seizure susceptibility. This could be due to the lack of modulatory control of inhibition consequent of imbalance of GABA and glutamate neurotransmitters upon kindling (Minamoto et al., 1992). In limbic epilepsy, tonic inhibition mediated by δ-containing receptors remains intact; moreover, GABA uptake by transport proteins also retains function (Pavlov and Walker, 2012; Pavlov et al., 2011), preventing accumulation of extracellular GABA that could increase inhibitory gain. Our reported decrease in endogenous tonic current during NSW suggests extrasynaptic receptors experience less inhibitory modulation due to the altered hormonal environment. Pathophysiological mechanisms and network aberrations may constrain $GABA_A$ receptor function in epilepsy (Cohen et al., 2003). The rapid kindling of the hippocampus in NSW mice produced an accelerated progression of epileptogenesis as well as development of sustained, excitable electrical activity that originated within the hippocampus. These results suggest that the hippocampus is more susceptible to excitability due to diminished AP. Fully kindled mice undergoing NSW displayed enhanced antiseizure sensitivity to the neurosteroid AP (**Figure 14A-B**), and lack of δ-subunit diminished neurosteroid sensitivity (**Figure 14C-D**). Therefore, plastic increase in δ-containing

$GABA_A$ receptors with high affinity for neurosteroids provides the opportunity for novel therapeutic targets to dampen excitability in individuals who are susceptible to seizures.

In conclusion, these results provide strong evidence that neurosteroid withdrawal, such as that which occurs during perimenstrual period, is associated with striking up-regulation of the extrasynaptic δ-containing $GABA_A$ receptors that mediate tonic inhibition in the hippocampus. This provides a molecular mechanism for enhanced anticonvulsant activity of neurosteroids and network inhibition in hyperexcitability conditions of catamenial epilepsy and other menstrual conditions linked to neurosteroid withdrawal. These findings provide a mechanistic rationale for neurosteroid therapy of catamenial epilepsy.

V.2 Neurosteroid Structure-activity Relationships at δ-subunit Extrasynaptic Receptors

The principal findings of the second specific aim uncover the structural significance of neurosteroid interaction with extrasynaptic $GABA_A$ receptors that provide tonic inhibition within the dentate gyrus. We examined an extensive library of neurosteroid structures and a wide concentration range to assess both physiological and clinically relevant potentiation of extrasynaptic receptors. The tonic current of DGGCs is highly dependent on δ-containing receptors (Stell et al., 2003; Glykys et al., 2008; Carver et al., 2014). We focused on the δ-subunit-specific tonic inhibition properties of native DGGCs in determination of SAR. Despite an inherent heterogeneity of the $GABA_A$ receptor population within native neurons, our findings support an obligatory role of δ-subunit in neurosteroid modulation of tonic inhibition. Synapse-localized receptors are less sensitive to neurosteroid modulation (Wohlfarth et al., 2002). Phasic and tonically active receptors differ in their GABA affinity (Mortensen et al., 2012), desensitization rate, agonist efficacy (Bianchi and Macdonald, 2002; 2003), and neurosteroid binding affinity (Brown et al., 2002; Wohlfarth et al., 2002). The differences in subunit composition properties are highly relevant to therapeutic use of neurosteroids. They are indicative of the complex neurosteroid interactions that occur within the brain that cannot be completely determined by heterologous expression systems in which physiological structure and receptor heterogeneity may be absent.

Previous studies have shown that greater levels of receptor expression in oocytes or HEK293 cells result in higher frequency of spontaneous $GABA_A$ channel opening (Hadley and Amin, 2007; Jensen et al., 2013). These properties signify artifacts of heterologous expression systems that could confound pharmacological activity profiles. Here we examine the spatially and functionally significant properties of $GABA_A$ receptor tonic current within the slice and hippocampal network. Using a model of δ-subunit deletion, we provide evidence of a biphasic effect of neurosteroid activity that results in increased inhibitory tone due to alternative receptor subtypes.

In the SAR studies, we used a range of concentrations that signify physiological and pharmacological levels. Aqueous concentration values of neurosteroid in perfusion reflect levels that are circulating within the extracellular fluid; however, the membrane concentration of steroid drives the binding interactions with $GABA_A$ receptors (Chisari et al., 2009). In examination of SAR within the slice, our findings reflect a gradual access of neurosteroid to receptors on the order of minutes rather than instantaneous or rapid access. Furthermore, our preparation permits possible accumulation of neurosteroid in surrounding tissue at the time of receptor modulation, analogous to that of a physiological state. Thus, the concentration-dependent responses we report here convey the pharmacodynamics activity and specificity of neurosteroids via allosteric modulation.

At higher concentrations, neurosteroids (> 1 μM) exhibit direct activation of $GABA_A$ receptors (Puia et al., 1990). The structural properties of neurosteroids that enable direct binding are not well understood. We described AP's capacity to directly activate extrasynaptic receptors by examining tonic current in WT and δKO DGGCs. AP was less efficacious in potentiating tonic current via direct receptor activation compared to allosteric binding with GABA. The exponential increase in tonic current due to application of 3 μM AP + GABA was greater than application of 3 μM AP alone in WT neurons ($p = 0.0046$). This indicates that the δ-subunit highly contributes to direct AP activation of tonic currents, as δ-containing receptors accounted for approximately 71% of the tonic current at 3 μM AP (**Figure 20C**). Previous report of cumulative $GABA_A$ receptor response indicates that high-efficacy/synaptic receptors are sensitive to direct activation by neurosteroid (Kokate et al., 1994). We could not extend such findings to tonic current recordings, as direct AP (0.1 - 1 μM) did not potentiate tonic current

from δKO DGGCs. Other data suggests that higher concentrations of neurosteroid may block the $GABA_A$ receptor channel pore, inhibiting chloride conductance (Brown et al., 2002). In contrast, we demonstrate extrasynaptic receptors displayed further potentiation at higher neurosteroid concentrations as well as direct activation of chloride channel conductance. A delayed response to the direct activation of neurosteroid has been previously observed (Kokate et al., 1994). However, due to the gradual bath perfusion of neurosteroid in interaction with the slice tissue, we were unable to detect any differences in the required time of channel modulation between allosteric and directly activating drug.

$GABA_A$ receptors have distinct gating properties in low-efficacy and high-efficacy states, and neurosteroids are uniquely sensitive to the channel function conferred by δ-subunit that enables continuous, inhibitory conductance (Bianchi and Macdonald, 2003). We found that neurosteroid activity on tonic currents to be δ-subunit dependent within DGGC. Deletion of δ-subunit completely diminished the allosteric potentiation and direct activation effects of AP. Therefore, the low-to-high efficacy transduction produced by neurosteroid is δ-subunit mediated under physiological conditions. These data support our previous findings of the significance of δ-subunit plasticity to tonic current function (Carver et al., 2014; Wu et al., 2013). Additionally, we discovered a role for other $GABA_A$ receptors which may contribute to tonic conductance in facilitation of high-efficacy states. Within δKO DGGCs, 10 μM GABA or ≥ 3 μM neurosteroid elicited modest hyperpolarizing, tonic responses. Despite δ-deficiency, the high-efficacy modulation signifies a biphasic response of the native receptor population in which non-δ receptors are being potentiated to influence tonic current. We have shown this potentiating response both in conditions of allosteric and direct neurosteroid activity. The subunit configuration and location of these high-efficacy receptors remains unclear, but may relate to the compensational shift of other subunit expression within the germline δKO mouse strain (Peng et al., 2002). Changes to neurosteroid sensitivity are also relevant to the hyperexcitability of epileptic granule cells (Kobayashi and Buckmaster, 2003) and the decreased expression of δ-subunit in epileptic hippocampus (Whissell et al., 2015). Large neurosteroid accumulation could overcome the fast desensitization of synaptic or perisynaptic receptors to provide for prolonged $GABA_A$ channel opening and increased hyperpolarizing tone. A large reduction in tonic conductance may lead to lack of shunting inhibition for δ-deficient neurons and likely presents a model for hyperexcitability within the hippocampus in which neurosteroids may still modulate

current at higher concentrations. A recent concatamer-receptor study reported that α4βδ and α1βγ2 $GABA_A$ receptors have largely similar structure-activity profiles for neurosteroids (Shu et al., 2012). We demonstrate SAR of neurosteroids that is specific to native, δ-containing receptors and further reveals a divergent role of tonic inhibition that is specific for the function of granule cells in the dentate gyrus.

Sex-dependent differences in $GABA_A$ receptor expression and function remain important to understanding network excitability and modulation by anticonvulsant drugs like neurosteroids (Galanopoulou et al., 2008ab). Our behavioral studies provide supporting evidence for sex-specific, distinctive receptor expression and plasticity within the adult brain. We used the 6-Hz model as diagnostic of a large array of structurally diverse neurosteroids, correlating their antiseizure effects with tonic function in the slice. In addition, we provide insight to comparative effects of neurosteroids in male and female mice *in vivo*, indicating differences in $GABA_A$ receptor substrate and extrasynaptic environment.

A previous report represented the GABAergic EC_{100} as the two-fold potentiation of GABA current by neurosteroid (Kokate et al., 1994). We ascertained the true extrasynaptic contribution of δ-subunit by parsing the contributions of δ- and non-δ-containing receptors in modulation of tonic current by GABA and neurosteroid. Relative efficacy of tonic current potentiation for our library of neurosteroids was compared for a wide range of concentrations (0.1 – 3.0 μM) to better assess structure-activity contributions to extrasynaptic, tonic current (**Table 5**). In addition, we developed a table to ascertain the correlation between neurosteroid modulation of tonic current inhibition and anticonvuslant profiles in the 6-Hz seizure model in mice (**Table 6**). We determined a strongly correlated relationship between the anticonvulsant potency of neurosteroids in protection of 6-Hz seizures and the capacity of neurosteroid to potentiate current of δ-containing extrasynaptic receptors.

Our experiments focused on neurosteroid SAR by experimentally isolating the extrasynaptic responses in native neurons within slice. This study reflects physiological modulation of localized, extrasynaptic receptors rather than measurement of aggregate responses, which are inclusive of all $GABA_A$ receptors within the neuronal membrane. All allosteric experiments were carried out in the presence of 1 μM GABA to compare neurosteroid concentration-dependent

current responses. AP was examined as a prototypical modulator of $GABA_A$ receptor function in comparison of other steroidal structures. The 3β-OH-modified neurosteroid (3β-AP) was unable to modulate extrasynaptic receptors, exhibiting a motif of inactivity for 3β-reduced steroids (Puia et al., 1990). The 3α-hydroxyl therefore has a critcal role in extrasynaptic receptor modulation. The α or β orientation of the C5 position affects the shape of the A ring of the steroid molecule (Hosie et al., 2007), and may influence receptor affinity. However, some previous studies using post-synaptic preparations report no change in the structure-activity relationship of 5β- compared to 5α-pregnane neurosteroids (Simmonds, 1991; Xue et al., 1997). In contrast, we observed significantly less tonic current modulation by 5β-AP stereoisomer, as AP maintained nearly 2-fold greater tonic current response compared to 5β-AP. These findings are consistent with prior reports of reduced 5β efficacy (Kokate et al., 1994; Hosie et al., 2006), but we reveal specific information for extrasynaptic receptor binding. Another report of neurosteroid modulation on δ-containing receptors had observed greater sensitivity to THDOC than AP (Brown et al., 2002). Interestingly, THDOC displayed significantly lower tonic current modulation compared to AP at sub-micromolar concentrations. ALFX exhibited reduced tonic current compared with AP, likely due to its ketone at the C11 region, which could interfere with the coordinated binding at the hydrophobic pocket in the transmembrane domain of the receptor. Thus, the conserved C3α and C17 groups may be influenced by other structural factors in extrasynaptic receptor interaction. Weaker tonic modulation by androstane neurosteroids indicates lower allosteric activation of tonic current. The androstane 17-β functional groups (see Table 3) suggest a more polar C17 position, resulting in weaker affinity for the hydrophobic binding pocket (Reddy and Jian, 2010). Structural differences in electronegativity stemming from the C17 position may therefore be crucial to the binding activity for extrasynaptic receptors. The ORG-20599 compound exhibited high extrasynaptic efficacy and affinity greater than AP. The morpholinyl conjugation in the A ring could drastically alter the shape of molecule and serve to facilitate the hydrogen bonding of the 3α group. However, ORG-20599 possessed very weak potency in 6-Hz antiseizure function for reasons possibly related to pharmacokinetics or ability to cross the blood-brain barrier, despite report as a potent anesthetic (Hill-Venning et al., 1996).

The site-specific residues determined to be crucial for neurosteroid binding were determined to be Q241 and N407/Y410 (Hosie et al., 2007). However, these studies were carried out in α1 subunit, and the α4 subunit similarities in interaction with the transmembrane binding pocket

remain unclear. A recent report incorporated T235W and Q240W mutations into the α_4 subunit and rendered δ-containing receptors (α4βδ) insensitive to positive allosteric modulation by AP (Jensen et al., 2013). Therefore, the higher affinity for the α_4 subunit binding interface and δ-dependent channel transduction greatly contribute to neurosteroid binding.

Based on the structure-activity data and integration of literature concerning extrasynaptic receptors, we designed a consensus pharmacophore model (**Figure 39**). Our proposed pharmacophore model suggests coordination of the two hydrogen bond donating groups in affinity for the transmembrane allosteric interface, facilitated by the δ-subunit. The proposed pharmacophore map is derived based on the correlation between neurosteroid modulation of tonic current and anticonvulsant profiles from the 6-Hz seizure model. The salient features of our proposed pharmacophore model are: a) The C3 and C17 regions remain critical to the δ efficacy of neurosteroids. b) The C5 region indicates a stereo-selectivity for neurosteroid interaction at extrasynaptic sites, similar to that of synaptic receptor binding. c) The C20 branch of the pregnane neurosteroids likely provides coordination of the hydrophobic pocket affinity that yields effects on both potency and efficacy. d) This potency is attenuated in the case of androstane neurosteroid binding of extrasynaptic receptors. e) Ketone group attached to the C11 position such as that in the synthetic neurosteroid alfaxalone proves detrimental to δ-subunit-containing receptor efficacy.

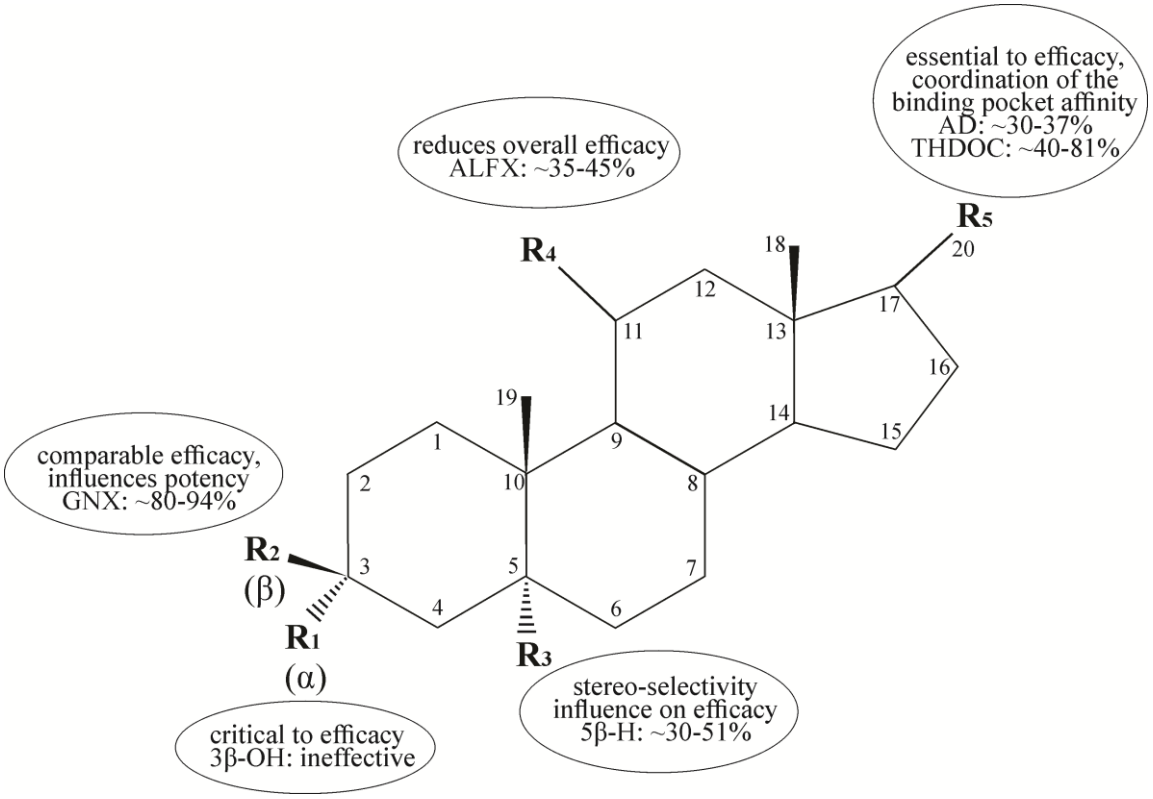


Figure 39. Pharmacophore map of neurosteroid structure-activity at δ-containing extrasynaptic $GABA_A$ receptors. The key structural components of the neuroactive-steroid chemical structure, based on structure-activity studies from Results section IV.2 and Table 5-6. These key structures are derived from electrophysiological and behavioral studies of neurosteroid activity on the putative extrasynaptic function of δ-containing $GABA_A$ receptors. R_1: C3-position stereoselectivity of the hydroxyl group. R_2: C3-position β-conjugation in conjunction with the 3α-hydroxyl group. R_3: C5-position stereoselectivity of the hydrogen bond upon 5α- or 5β-reductase activity. R_4: C11-position conjugation of double-bonded oxygen in the case of alfaxolone and alfadalone synthetic neurosteroids. R_5: C17-position β-conjugation necessary for efficacy, C20-position ketone for pregnane neurosteroids (AP, THDOC), or C17-position for androstane neurosteroids (AD, AN, ETIO). Values listed are approximate range of neurosteroid potentiation of I_{tonic} as a percentage of the AP prototypical I_{tonic} responses in electrophysiology slice recordings.

V.3 Role of δ-subunit in Seizure Susceptibility and Epileptogenesis

The principal finding for this study is that mice with a targeted germline deletion of δ-subunit in the brain exhibit increased propensity for the development and persistence of kindling epileptogenesis, indicating a crucial role for δ-containing receptors in seizure susceptibility. We observed a markedly increased rate of kindling epileptogenesis, afterdischarge duration, and seizure severity within δKO mice. In addition, we found sex-specific differences in kindling epileptogenesis. Male WT and δKO mice exhibited more rapid kindling and persistence of seizures than their female genotype counterparts. Previous studies have indicated sex-specific distinctions in seizure propensity and epilepsy (Galanopolou, 2008ab; Pack et al., 2011; Reddy, 2009b; 2011; Reddy and Kulkarni 1999; Wu et al., 2013). These differences span a wide range of properties, including expression of $GABA_A$ receptors as the substrate for network inhibition, as well as neuroendocrine pathophysiology that may promote conditions of seizure susceptibility. Our findings suggest that alterations in δ-containing extrasynaptic receptors contribute to the balance of excitatory inputs in the hippocampus and may be key mediators of epilepsy pathophysiology. Collectively, our functional and behavioral observations indicate that epileptogenesis is facilitated in conditions of attenuated tonic current in the dentate gyrus, attributed to lack of δ-containing extrasynaptic receptors.

The hippocampus is a key structure in epilepsy pathophysiology. As the modulatory "gate-keeper" and filter for the hippocampus, the dentate gyrus possesses inhibitory control over excitatory inputs from the entorhinal cortex and other brain regions (Coulter and Carlson, 2007; Heinemann et al., 1992). The putative function of tonic inhibition in the hippocampal principal cells is achieved by shunting of excitatory current, thereby altering the slope of the input-output relationship as divisive inhibition (Mitchell and Silver, 2003; Wlodarczyk et al., 2013). Tonic chloride conductance also mediates the baseline hyperpolarizing membrane current via subtractive inhibition. However, the function of δ-containing extrasynaptic receptor modulation at the network and systems level remains poorly understood. Our results from behavioral kindling of the hippocampus in δ-deficient mice provide a better understanding of the role of extrasynaptic receptors in the epileptogenic pathways.

Several caveats regarding interpretation of the findings are of consequence to these behavioral studies, in which the proper context must be considered. First, the results apply only to the hippocampus kindling model used in these studies. The kindling model, while sharing many similarities with human complex seizures, cannot accurately or reliably account for all idiopathic forms of epilepsy. Therefore, better understanding as to the underlying causes of seizure progression will help to more closely link the kindling model to human epileptogenesis in translational studies. In an effort to recapitualate the genotypic differences brought on by lack of δ-subunit, we were unable to show similarly increased seizure susceptibility in δKO mice with the PTZ chemoconvulsant model. Secondly, the knockout animals used in these experiments possess unique qualities that could represent epiphenomena related to the transgenic manipulation that results in compensatory receptor expression and other changes (see section I.8). Moreover, we uncovered developmental differences between WT and null mutants, indicating the possibility of brain development alterations leading to divergent phenotypes in addition to loss of δ-subunit function. Nevertheless, we provide strong evidence for the contribution of δ-subunit to seizure modulation of the kindling model. Conditional transgenic and translational models will be useful in future directions to study network control of excitability and epileptogenesis.

In the kindling model, there was no observable change in the baseline behavioral seizure activity during the early stimulations, and the electrographic afterdischarge threshold was simliar between genotypes. Focal electrographic afterdischarge is required for the induction of epileptogenesis in the kindling model (Goddard et al., 1969). The accelerated kindling development in δKO mice could be attributed in part to the differences in focal electrographic seizures, as the duration of evoked afterdischarge duration displayed disparity among WT and δKO during the early stimulation sessions. There was a reliable correlation of kindling rate/progression of seizures and afterdischarge duration. However, as kindling progressed to later stages of seizure activity, afterdischarge duration tended to normalize among genotypes. Hyperexcitability was reinforced with recurrent electrical stimulation, resulting in a steeper slope of progression for δKO mice compared to WT. These results indicate that the structural and functional connections required to exhibit behavioral and electrographic seizures may be altered in δKO mice. Seizure propogation originated in the hippocampus, and we conclude from our

functional studies that this region has significant alterations in network excitability due to germline knockout.

The mechanisms underlying epileptogenesis involve an interaction of acute and delayed anatomic, molecular, and physiological events that are complex and multifaceted (Walker et al., 2002). Neuronal injury activates diverse signaling events, such as inflammation, oxidation, apoptosis, neurogenesis, and synaptic plasticity, which eventually lead to structural and functional changes in neurons (Löscher, 2002; Pitkänen et al., 2009). In addition, information is emerging concerning extrasynaptic inhibition alterations due to seizures, injury, and excitotoxicity (Cohen et al., 2003; Dibbens et al., 2004; Pandit et al., 2013; Pavlov et al., 2011). These changes are eventually manifested as abnormal hyperexcitability and spontaneous seizures. Reduction to GABAergic tonic inhibition that typically facilitates shunting of excitatory current could be a reasonable mechanism for the promotion of epileptogenesis in δKO mice, along with other precipitating conditions.

To assess specific modulation of tonic inhibition we administered low pharmacological dose of AP neurosteroid to mice that did not result in any sedative, anesthetic, or motor toxicity effects. This dose was studied in prolonging the progression of seizures, but it was not effective in preventing the full kindling of maximum seizure severity. Overall, neurosteroid treatment retarded epileptogenesis compared to vehicle-injected controls in both WT and δKO mice. However, the disease-modifying effects of neurosteroid were diminished in δKO animals in later stages of kindling development. The delay in anticonvulsant effect over late-stage kindling of δKO is significant in the therapeutic control of seizures in which individuals with greater susceptibility to seizures and hyperexcitability due to brain insult are resistant to conventional antiepileptic drugs. We show that δKO exacerbation of repeated electrical stimulation is less sensitive to the antiepileptogenic effect of the neurosteroid AP. This may provide a framework for understanding neurosteroid anticonvulsant retardation of epileptogenesis in epilepsy models.

In this study, we demonstrate that δ-containing receptors play an important role in the development and persistence of limbic epileptogensis. These findings suggest extrasynaptic $GABA_A$ receptors may serve as a novel target for therapeutic modulation of inhibition and may provide unique approaches to prevent epileptogenesis. Additional understanding of the

molecular mechanisms underlying expression of δ-containing receptors may provide better insights on how dentate gyrus tonic inhibition influences neuronal excitability in the context of a variety of pathophysiological conditions. We have also provided a behavioral framework for understanding the developmental and cognitive differences of the germline δ knockout in the support of seeking better conditional transgenic models for understanding the role of δ-subunit in the hippocampus.

CHAPTER VI
CONCLUSIONS

This work defines a clear role for δ-subunit-containing extrasynaptic receptors in mediation of tonic inhibition and seizure susceptibility. The extrasynaptic receptor class within the dentate gyrus represents an important target for novel therapeutic drugs that could control brain excitability. We have identified a molecular mechanism of catamenial epilepsy by exploring increases to extrasynaptic receptor expression and function using an animal model of perimenstrual-like neurosteroid withdrawal. This withdrawal model provides a rationale underlying neurosteroid therapy in treatment of catamenial epilepsy conditions, whereby tonic inhibition is the functional target by neurosteroid modulation of δ-subunit $GABA_A$ receptors. As investigation of neurosteroid pharmacology continues, new agents that are more specific in binding to extrasynaptic $GABA_A$ receptors could help to further identify the allosteric and direct binding of neurosteroids. The lipophilic nature of neurosteroids denotes more complex lateral binding on the receptor structure. In our approach, we have outlined the structure-activity relationship of neurosteroids at δ-containing extrasynaptic receptors in native neurons within the hippocampus. We have identified key structural features that provide strong efficacy for neurosteroid potentiation of tonic current inhibition. This research also determines a role for δ-subunit in network excitability by hippocampus epileptogenesis with *in vivo* mouse models. We have provided a framework that confirms δ-subunit-containing $GABA_A$ receptors as a functionally significant target of neurosteroids in the promotion of network inhibition using the germline δKO mouse model. Due to the compensational alterations to $GABA_A$ receptor expression in the germline δKO mouse, it remains possible that the behavioral and physiological profiles of the model are indirectly altered (Stell et al., 2003). Regional and temporal control of δ-subunit expression in mice would aid in clarifying the role of extrasynaptic receptors in the brain. Future directions include seeking to further resolve the functional significance and plasticity of δ-containing, extrasynaptic receptors in epilepsy, stress, and other pathophysiological states in which neurosteroid interaction could play a significant role.

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